ANNUAL REPORT
OF
PROGRAM ACTIVITIES
NATIONAL INSTITUTE OF DENTAL RESEARCH
FISCAL YEAR 1979
PART IV

U. S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE
Public Health Service — National Institutes of Health

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United States. National Institute of Dental
Research.

Report of program activities.

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PART V - COLLABORATIVE RESEARCH PROJECTS

REPORT OF THE DIRECTOR OF INTRAMURAL RESEARCH

THE NATIONAL INSTITUTE OF DENTAL RESEARCH

October 1, 1978 - September 30, 1979

The scope of research in the Intramural Program encompasses many different fields of biomedical science as can be perceived from reading the summary statements of the Laboratory and Branch Chiefs as well as the individual project reports. These summary statements show that much of the effort is concerned with seeking answers to fundamental questions in order to design methods and procedures for prevention and treatment of diseases and disorders. These studies contribute knowledge that is of general importance to scientists studying diseases not only of the mouth but of other parts of the body. Animal models are used widely while other experiments are performed in vitro using animal organs, tissue cells and cell components or clinical samples. Experiments are also carried out with synthetic compounds. Investigative disciplines presently represented are those of biochemistry, biology, biophysics, chemistry, immunology, microbiology, neurophysiology, and virology.

Last year the Institute initiated a search for a new Clinical Director. The search, continued this year, was extended to countries outside the United States. In December, 1978, Dr. Karl-Åke Omnell, Professor, School of Dentistry, Lunds Universitet, Malmo, Sweden, was offered and accepted the position. He is expected to come on duty late in the Fall of 1979.

The Institute is indeed fortunate to have a person of his caliber and standing join the staff. As Clinical Director, Dr. Omnell will bring leadership and direction to our clinical dental research programs and help narrow the gap that has existed between clinical and basic science disciplines.

One of the principal factors which made it very difficult to attract qualified candidates for the position of Clinical Director was a limitation on the number of positions that the Institute could make available to develop new and expanded program efforts in the clinical area. The reduction in our full-time ceiling of 12 which was imposed on the Institute this year has further handicapped our efforts and expectations in this direction. Moreover and just as critical, the Institute's existing basic sciences laboratories have had to absorb not only the cost of the 12 positions but also have had to come up with the four positions committed as a minimum to the new Clinical Director. This total loss of 16 positions out of a research staff which totaled 145 represents a cut of over 11% in our basic research efforts which have been and continue to be outstanding, highly productive, and in several instances unique to NIH.

The position reduction coupled with lack of increases in funds, in spite of rampant inflation, mean that the Institute's Intramural Program is rapidly losing ground. It is no longer a question of no-growth. It is a question of severe cut-backs, even of survival. Unless something can be done to stop this trend, the Institute and NIH may shortly lose several of its highly trained and most talented employees. It should not be necessary here to emphasize the uniqueness and advantages of the Intramural Programs at an institution like NIH. We need to do everything possible to insure that they continue to be viable and productive.

The ceiling reduction has also affected another of the Institute's new initiatives adversely. Thus, last year the Institute offered, for the first time, two-year appointment for Clinical Dental Associates under the NIH Associates Program. Initial plans were for the selection of two candidates each year for a total of four. However, only one candidate was appointed last year primarily because of lack of qualified applicants. This year with early distribution of the NIH Associates Program booklet the Institute expects many more applicants but may not be able to select any of them because of the ceiling cuts.

The Institute continues to experience difficulties in attracting qualified minority doctoral candidates to its Intramural Programs, difficulties that are further complicated by the loss of positions. A number of minority candidates were given personal interviews and were urged to apply to the Clinical Dental Associate Program. None did. One dentist with extensive clinical experience was approached regarding a position in the Dental Services Section. He declined. The position was filled, however, by a woman Commission Officer, a first for the Institute. Progress with respect to the scheduling of visits by staff members to minority institutions is reported by the Institute's EEO Coordinator.

A new Section of Proteoglycan Biochemistry was created by the Director of the Institute in the Laboratory of Biochemistry and Dr. Vincent Hascall of the Laboratory was appointed Chief of the new section. These actions which were originally requested by the Chief of the Laboratory were endorsed by the Board of Scientific Counselors following a review of the Laboratory last year.

In September, 1979, the Intramural Research Program sponsored a highly successful four and a half day Conference on Basic Mechanisms of Cellular Secretion. The Conference organized by Drs. A. Hand and C. Oliver, Laboratory of Biological Structure, was attended by over 200 people, many of whom were from foreign countries. In addition, representatives of most of the other Institutes at NIH and many other Government Agencies participated. The meeting summarized current knowledge of protein secretory mechanisms and their regulation, addressed new problems, and defined new approaches

The eight-person Board of Scientific Counselors participated in three separate program reviews. Ad hoc members were added to each review group to provide greater scientific expertise. In September, the complete Board met for a day and a half to discuss the individual reviews in details and make recommendations to the Director of the Institute and the Director of Intramural Research.

In fiscal 1979 the following NIDR Staff members received awards and/or recognition for their contributions to the Institute's program: DHEW Distinguished Service Award - Dr. Marie U. Nylen; NIH Director's Award - Dr. James Bosma; NIH Merit Award - Dr. Vincent C. Hascall, Mr. Guy R. Hawkins, Ms. Leta Stevenson; NIH EEO Special Achievement Awards - Ms. Barbara Corcoran, Ms. Mary Ann Williamson; Awards from Organizations Outside USPHS-NIH: American Association of Oral and Maxillofacial Surgeons Research Recognition Award - Dr. Edward Driscoll; Commanding Officer's Annual Award for Civism, National Naval Dental Center - Dr. Paul H. Keyes; Alumni Merit Award, Creighton University, Omaha, Nebraska - Dr. Charles L. Wittenberger;

Institute staff made frequent appearances as invited speakers, session chairmen, and members of Program Committees at both National and International Meetings, Workshops and Symposia. The following merit special mention: Honorary Lecturer: Fourth Annual Andrew Mark Leppard Memorial Lecture, College of Physicians and Surgeons, Columbia University - Dr. Abner L. Notkins; Invited Speaker: University of Copenhagen - 500 Years Jubilee Symposium - Dr. Abner L. Notkins; Member, Scientific Program Committee, Third World Congress on Pain, to be held 1981 - Dr. Ronald Dubner; Member, Scientific Program Committee, International and American Association for Dental Research - Dr. Marie U. Nylen.

Report of the Clinical Dental Services Section
Office of the Director of Intramural Research
National Institute of Dental Research
1979

The Clinical Dental Services Section furnishes dental consultative services and dental therapies for Clinical Center patients of all Institutes, originates and carries out its own research projects and provides support and facilities for clinical programs sponsored by the Dental Institute's laboratories and branches.

The Clinical Dental Services Section had an accreditation review on April 4, 1979 by a representative of the Council on Hospital and Institutional Dental Services, American Dental Association. Collaborative research projects were reviewed by Scientific Counselors, NIDR on April 5, 1979.

During the past year a cooperative effort between this section and the Department of Dental Hygiene, Baltimore College of Dental Surgery, University of Maryland has led to senior student participation in Dental Clinic patient education and orientation programs. Students may select this assignment as additional training in their Community Service Practicum-Dental Hygiene Externships.

Active research projects initiated by Section personnel include:

Effect of various preoperative and postoperative rinses on healing after oral surgery (78-D-89) Z01 DE 00241-02 IR. This study evaluates the effectiveness of four topical solutions to reduce localized osteitis and infection---two adverse sequelae associated with tooth extraction. The solutions (1) chloramine-T, 1%; (2) sodium bicarbonate, saturated solution; (3) normal saline, 0.9% and (4) povidone-iodine, (1% available iodine) are used twice daily by the patient as topical antiseptic mouthrinses.

Psychological and radiographic evaluations of the orthognathic surgery patient (76-D-249) Z01 DE 00213-03 IR. This study allows an evaluation of the presurgical and postsurgical psychological and functional aspects of individuals with developmental osseous defects of the face and jaws. The psychological impact of the surgical correction and postoperative alterations in bone and tooth position are being studied.

The potential of a fluoride mouthrinse to alleviate xerostomia symptoms (76-D-278) Z01 DE 00217-03 CI C. Preliminary tests indicated that a sodium fluoride mouthrinse (1.23%F) transiently increased the volume of whole saliva in normal subjects. Recent studies demonstrated that the fluoride mouthrinse produced a statistically significant increase in saliva volume over baseline when administered to subjects xerostomic due to radiation therapy, chemotherapy and Sjogren's syndrome.

An evaluation of periodontal pocket reduction surgery and the topical application of antibacterial agents in the control of periodontitis (78-D-78) Z01 DE 00239-02 IR. This study compares the effectiveness of two home applied oral hygiene regimens in removing subgingival tooth accumulated bacteria. Either conventional mechanical techniques alone or mechanical techniques in conjunction with topically applied sodium bicarbonate-sodium chloride "paste" are prescribed to periodontitis patients who have had pocket reduction surgery performed in one-half of their mouths.

The Clinical Dental Services Section's collaboration with NIDR laboratories and branches involve the following projects:

Laboratory of Microbiology and Immunology

Investigation of cellular immunological mechanisms in periodontal disease and the correlation between periodontal disease and histocompatibility antigens (76-D-330) Z01 DE 00205-03 LMI.

Investigation of the etiology and the extra-salivary complications of Sjogren's syndrome (75-D-54) Z01 DE 00085-06 LMI.

Observations on the periodontal microbiota of patients with active periodontal lesions and with lesions controlled by personal oral hygiene and tetracycline therapy (78-D-77) Z01 DE 00096-06 LMI.

Laboratory of Neurobiology and Anesthesiology

A psychophysiological evaluation of the effectiveness of (and recovery from) intravenous sedation for oral surgery procedures in ambulatory patients (76-D-148) Z01 DE 00132-05 NA.

Assessment and measurement of experimental and clinical acute pain in a dental situation including an evaluation of known pharmacological and non-pharmacological agents used in pain control (75-D-59) Z01 DE 00133-05 NA.

Effects of chronic electric stimulation of the brain and narcotic analgesics on human responses to noxious and non-noxious stimuli: Clinical and experimental evaluations (75-D-4) Z01 DE 00784-02 NA.

Clinical Investigations Branch

Taste and its disorders Z01 DE 00212-03 CI.

Oral pharyngeal lipase (74-D-13) Z01 DE 00218-03 CI.

The development and evaluation of improved dental radiograph systems, with emphasis on factors influencing diagnostic performance Z01 DE 00065-08 CI.

Laboratory of Oral Medicine

The relationship of mechanical trauma to oral aphthous ulcers (77-D-97) Z01 DE 00094-06 LOM.

Two publications, not included in individual progress reports, have resulted from collaborative clinical research projects by this section--(1) J.J. Baker, W.E. Wright, S.P. Chan and J.J. Oppenheim: Longitudinal Effects of Clinical Therapy and the Edentulous State on the Transformation of Lymphocytes from Patients With Severe Periodontitis. Clin. Exp. Immunol. (Nov. 1978) 34, 199-205. (Collaborators: Litton Bionetics, Kensington, Md. and LMI, NIDR, NIH (2) B.J. Baum and W.E. Wright: Demonstration of Fibronectin as a Major Extracellular Protein of Human Gingival Fibroblasts. J. Dent. Res., in press. (Collaborators: Pulmonary Branch, National Heart, Lung and Blood Institute, NIH).

The Clinical Dental Services Section continues to offer a unique and essential opportunity to provide a link between basic research laboratory activities, clinical research laboratory activities and the clinical human disease problem.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE
PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF
HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DE 00213-03 IR

PERIOD COVERED
October 1978 to September 30, 1979

CT 060123

TITLE OF PROJECT (80 characters or less)
Psychological and Radiographic Evaluations of the Orthognathic Surgery Patient

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	James B. Sweet	Dental Director	IR	NIDR
OTHER:	John Folio	Chief Clin Dental Ser Sect	IR	NIDR
	Richard H. Gracely	Research Psychologist	NA	NIDR
	Harold A. Greenberg	Chief Clinical Care	DCBR	NIMH
	James Madero	Clin Care Cons Psychologist		NIMH
	Alice A. Macynski	Clinical Nurse (General)	IR	NIDR
	Donald P. Butler	Oral Surg-Anesthesiologist	NA	NIDR

COOPERATING UNITS (if any)
None

LAB/BRANCH
Office of the Director of Intramural Research

SECTION
Clinical Dental Services Section

INSTITUTE AND LOCATION
NIDR, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: .35	PROFESSIONAL: .30	OTHER: .05

CHECK APPROPRIATE BOX(ES)
☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)
The psychological and anatomical changes that take place in individuals with middle and lower facial bony defects and malocclusions of the teeth after orthognathic surgery are measured and evaluated. Pre-and post surgery data obtained by tests, interviews, radiographs, photographs and orthodontic study casts taken at specific time intervals for a period of two years postoperatively are compared.

PHS-6040
(Rev. 10-76)

Project Description

I. Objectives:

Many individuals with middle and lower facial bony deformities or severe malocclusion require surgical correction in addition to routine orthodontic treatment. This includes patients with Class II or Class III malocclusions, patients with apertognathia, and patients with severe facial asymmetry.

The preoperative psychological state of these patients is influenced by their facial appearance, functional disability, and by expected postsurgical changes.

Regression and relapse are common postoperative complications associated with orthognathic surgery.

The specific aims of this study are to establish the psychological impact of the deformities prior to surgery, to determine the expectations from surgery and to correlate the findings after a 2 year follow-up period. Postoperation changes in facial bone and tooth positions which may occur are also being examined.

II. Methods Employed:

1. Patients between 15 and 45 years of age who have malocclusions that can be corrected by surgery, or a combination of surgery and orthodontics are examined and selected for study.

2. Preoperative photographs, study models and panoramic and cephalometric radiographs are taken on each patient.

3. Preoperatively, each patient and a family member is interviewed by a psychiatrist. Personality variables are assessed by standard psychological tests, and preoperative expectations are assessed by a questionnaire.

4. Surgical procedures are performed in the operating room under general anesthesia using commonly accepted techniques.

5. At 3 months, 12 months, and 24 months following surgery the battery of psychological tests are readministered and the patient is reinterviewed by the psychiatrist. At this time, resolution of presurgical expectations and overall satisfaction of the procedure will be determined.

6. Panoramic and cephalometric radiographs, study models, and facial photographs will be taken at the 3, 6, 12 and 24 month intervals to assess postoperative stability.

7. At completion of the study a final evaluation and comparison of the results will be done using conventional biostatistical methods.

III. Major Findings:

To date 15 subjects are in various stages of therapy. No findings are available for reporting since this project entails long term post-surgical evaluations.

IV. Significance to Biomedical Research and the Program of the Institute:

The investigation may increase our understanding of personality and psychological profiles in orthognathic surgery patients. This will allow a more thorough pretreatment evaluation and a more realistic definition of the patients expectations, which will result in our providing a better service to the patient.

V. Proposed Course:

Continue until approximately fifty patients have been completed.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 DE 00239-02 IR

PERIOD COVERED
October 1, 1978 to September 30, 1979

CT 060122

TITLE OF PROJECT (80 characters or less)

Evaluation of Surgery and Antibacterial Agents in Control of Periodontitis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	William E. Wright	Sr. Staff Dentist	IR	NIDR
Other:	Paul H. Keyes	Dental Director	LMI	NIDR
	Surya A. Howard	Health Technician (Dental)	LMI	NIDR
	Marjorie L. Meehan	Health Technician (Dental)	IR	NIDR

COOPERATING UNITS (if any)
None

LAB/BRANCH
Office of the Director of Intramural Research

SECTION
Clinical Dental Services Section

INSTITUTE AND LOCATION
NIDR, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: .25
PROFESSIONAL: .20
OTHER: .05

CHECK APPROPRIATE BOX(ES)
☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The effectiveness of conventional mechanical hygiene alone and in combination with topical applications of sodium bicarbonate-sodium chloride "paste" to control accumulations of oral bacteria will be compared in subjects with moderate periodontitis. Each subject will also undergo periodontal pocket reduction surgery in two of four posterior quadrants to allow assessment of this commonly used mode of periodontal disease therapy. Evaluation criteria will be (1) motile microscopic forms and white blood cells obtained from periodontal pockets will be viewed and numerically estimated by phase microscopy, (2) gingival sulci bleeding index and (3) measurements of gingival attachments relative to the cemento-enamel junction and gingival margin.

Project Description

I. Objectives:

The purpose of this clinical research project is to compare the effectiveness of two home applied oral hygiene regimens in removing subgingival tooth accumulated bacteria...(1) conventional mechanical techniques-brushing, flossing and water irrigation versus (2) the same conventional mechanical techniques in conjunction with topical applications of a sodium bicarbonate-sodium chloride "paste". Periodontal pocket reduction surgery will be performed in two of four posterior quadrants of all subjects selected for this study with the diagnosis of moderate generalized periodontitis.

Combinations of surgical areas versus nonsurgical areas in conjunction with one of the two home therapy regimens will be evaluated as to their role in effectively assisting the patient to prevent or control progressive destruction of the periodontal attachment apparatus.

II. Methods Employed:

1. Male or female patients ranging in age from 20-49 years and demonstrating moderate generalized periodontitis will be selected for study.

2. Pre-study baseline periodontal parameters will be recorded. Evaluation criteria will include (1) motile microscopic forms and white blood cells obtained from subgingival plaque scrapings will be numerically estimated by phase microscopy. (2) gingival sulci bleeding index and (3) measurements of gingival attachments relative to the cemento-enamel junction and gingival margin.

3. Subjects will be randomly assigned to perform one of two oral bacterial control regimens (conventional mechanical only or conventional mechanical plus daily topical applications of an "antimicrobial salt paste"). Random selection of two posterior quadrants to receive periodontal pocket reduction surgery will be used for all subjects.

4. Criteria evaluations will be made before treatment, after surgery one month and at three month intervals throughout the three year study.

5. Patient achievement goals will be an appreciable reduction in the numbers of motile bacterial forms and white blood cells in their subgingival plaque scrapings as determined by phase microscopy. Also a reduction in gingival bleeding

index to 10% of total sites tested will be a performance goal.

6. Prevention or control of progressive loss of gingival attachment as indicated by serial pocket measurements over a three year period will be evaluated.

III. Major Findings:

No reportable findings, patients still being selected and evaluated.

IV. Significance to Biomedical Research and the Program of the Institute.

This investigation will give insight into the types of therapy that can be most effective in controlling the common disease, chronic periodontitis. Information should be provided on the potential for treating certain periodontal lesions without surgical intervention. A conservative approach would be of value in treating the great numbers of individuals afflicted with chronic periodontitis for whom surgical therapy is not feasible due to lack of finances, facilities and trained clinicians.

V. Proposed Course:

To evaluate a minimum of 12 subjects as outlined to see if statistical significance can be attributed to control regimens.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 DE 00241-02 IR

PERIOD COVERED
October 1, 1978 to September 30, 1979 CT 060124

TITLE OF PROJECT (80 characters or less)

Effect of Various Preoperative and Postoperative Rinses on Healing After Oral Surgery

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	James B. Sweet	Dental Director	IR	NIDR
OTHER:	Alice A. Macynski	Clinical Nurse	IR	NIDR
	Donald P. Butler	Oral Surg-Anesthesiologist	IR	NIDR

COOPERATING UNITS (if any)

None

LAB/BRANCH
Office of the Director of Intramural Research

SECTION
Clinical Dental Services Section

INSTITUTE AND LOCATION
NIDR, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.9	0.5	0.4

CHECK APPROPRIATE BOX(ES)

x (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

(a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This clinical study is designed to allow the investigators to evaluate the effectiveness of four different solutions used topically before and after oral surgery procedures. Included will be normal saline solution, the conventional rinse, which will serve as a control. Postoperative localized osteitis and infections will be recorded if they occur, as data, to be considered in the overall evaluation of the use of topical solution for possible prevention of these postoperative problems.

PHS-6040
(Rev. 10-76)

Project Description

I. Objectives:

The overall incidence of localized osteitis throughout the mouth has been reported from 0.9% to 3.2%. The incidence of localized osteitis in third molar sites has recently been decreased with the use of lavage technique from 20-30% to under 6%, and the infection rate to under 3%. This small percentage of patients, however, still suffer with extreme postoperative pain. Conventionally, patients rinse with a weak saline solution the day following surgery and continue for a few days postoperatively. The saline is used primarily to mechanically clean the extraction wound of any debris present; it will additionally reduce the presence of bacteria. Various other preoperative and postoperative topical solutions have been tried with variable success. Legarth and Munster-Swendsen recently used a chlorhexidine solution for postoperative rinsing of third molar extraction sites and achieved a 45% reduction in the incidence of localized osteitis in a study involving 415 cases of mandibular third molars. Another recent study with a preoperative rinse of chloramine-T reduced the incidence of localized osteitis in mandibular third molar sites from 4% to 1.6%. It is thought that the use of certain antiseptic mouthrinses could achieve similar or possibly better results, especially if used both pre- and postoperatively.

The purpose of this project is to clinically test three different solutions along with the conventional saline usedpre- and postoperatively with oral surgery procedures to compare the incidences of localized osteitis and infection rate to determine if there are advantages with the use of other solutions as a rinse.

II. Methods Employed

An informed consent will be signed on GSA Standard Form 522, and a specific informed consent for this study will also be signed.

Immediately before the surgical procedure, the patient will rinse with 10 ml of a solution of either (1) 0.9% sodium chloride (normal saline), (2) 1% chloramine-T, (3) povidone-iodine (1% available iodine), or (4) saturated sodium bicarbonate for a period of one minute. The surgery will then be performed in a normal manner. The patient will receive both oral and written postoperative instructions for use of the mouthrinse for four to six days after surgery. Ten milliliters of the same solution used preoperatively will be used for one minute both morning and evening; 120 ml will be dispensed for the patient's use during this period of time. The patient will begin the rinses the day following surgery and continue their use until his scheduled postoperative observation appointment when sutures will also be removed. The patient will be examined for signs of localized osteitis or infection five days postoperatively, except when the fifth day is on a

Saturday or Sunday. In these cases the patient will be examined either on the fourth or sixth day postoperatively.

Specific criteria for evaluation of a patient with localized osteitis will be: (1) a severe pain in the alveolus of a surgical site two to four days postoperatively, (2) the presence of a foul, grayish exudate from the surgical site, (3) the presence of a necrotic odor from the surgical site, or (4) observation of a denuded, bony alveolus. At least two of these criteria will be present for a diagnosis of localized osteitis to be made.

Specific criteria for evaluation of a patient with an infection will be: (1) a swelling which persists, increases, or appears after four to five days postoperatively, (2) a purulent drainage from the surgical site, (3) a pulse rate significantly increased above the normal for the patient and persistently so elevated, or (4) a fever usually above 38.3°C, which remains elevated even when the patient remains well hydrated. At least one of the above criteria will be present for a diagnosis of postoperative infection to be made.

If an infection or localized osteitis occurs it will be treated and recorded as being present. Systemic antibiotics will not routinely be used postoperatively, except where infection occurs, and in those cases, they will be employed immediately when the diagnosis is made.

After five days (or if the fifth day is on Saturday or Sunday, then either four or six days) the surgical sites will be observed for healing progress. This data will be recorded with the patient's postoperative data sheets. The patient will then be discharged with follow-up appointments if needed. The patient will be instructed to return if he develops any further postoperative problems.

III. Major Findings:

The project was initiated in June 1978, thus insufficient data is available for reporting at this time.

IV. Significance to Biomedical Research and the Program of the Institute:

The results of this study can provide important information relative to a good topical rinse for use after oral surgery procedures. Positive results will allow the recommendation of a topical rinse to oral surgeons and dentists for use in prevention of certain postoperative oral surgery problems.

V. Proposed Course:

Continue project until sufficient data is generated to compare results with previous studies.

Report of the Microbial Systematics Section

National Institute of Dental Research

The Microbial Systematics Section is charged with establishing a data bank for information describing diverse strains of microorganisms. Special emphasis is placed on the human oral microbiota. For this purpose, collaborative projects are on-going with microbiologists distributed throughout the world.

At present there are tens of thousands of scientists, physicians, public health personnel, and others involved in some aspect of microbiology. The number of microbial strains isolated, characterized, and (in many cases) preserved, by individuals runs into the millions. Hundreds of millions of bits of information have been developed on these strains. However, these data are not resident in a single, centrally located system, permitting rapid and efficient utilization. Because of the large volume of information involved and because, in several applications such as classification and identification, mathematical manipulations of the data are required, electronic processing of these data is necessary.

In collaboration with personnel of the American Type Culture Collection; the Food and Drug Administration; the Center for Disease Control; the Veteran's Administration and numerous academic microbiologists, strain data are being entered into the data bank which provides such services as: data on specific organisms and/or groups of organisms, location of strains with special characteristics, identification of unknown isolates, cluster analysis definition of parameters of taxa, data management and report writing aids for research purposes, aids in quality control of tests, methods, and laboratories, and communication of data via common format.

Data files of primary data on a large number of microorganisms found in the oral cavity and related types are established. These files provide a resource for asking both ecological and epidemiological questions of interest in dental research.

Programs have been developed and tested to enter, retrieve, and analyze the data in a variety of ways for epidemiological, diagnostic, taxonomic, ecological, etc., uses. The long term goal is to establish a world-wide data bank at a series of cooperating centers. As experience grows, better programs are being designed and implemented.

The system originally developed for bacteria is now being expanded to include the yeasts, molds, algae and protozoa. A series of monographs describing the expanded system is in varying stages of preparation.

Extensive files of descriptions of filamentous and pleomorphic organisms are being assembled. The files cover all the described types of *Mycobacteria*, blend into the *Nocardia*, then through the *Actinomycetes* (especially a unique set on oral isolates), and finally, *Bacterionema*. An extensive cooperative study has been initiated to study the oral pleomorphic bacteria (many of which are associated with disease). The study will provide a standard set of well characterized bacteria for the Dental Research community. The data from this study will be incorporated into the files on pleomorphic organisms. These files are being actively used in collaboration with the submitters of the data as well as numerical taxonomists to revise the badly confused taxonomic relationships of these bacteria. Such revision is necessary to avoid the misidentification (leading to erroneous epidemiological conclusions) which are found in some recent dental research literature.

Other files on non-filamentous oral organisms (streptococci, lactobacilli, veillionella, etc.) are being constructed to study correlations among caries activity, phenetic span of characters, serology, source of isolation, and host descriptions.

One of the long term goals in establishing all these files are the establishment of probability tables to allow computer-aided probabilistic identification of oral isolates. Probability matrices, for on-line identification of bacteria (including Gram negative rods, lactobacilli, streptococci, bacilli, etc.) have been constructed. They are available to research workers for field use.

PUBLICATIONS:

Kaneko, T., Hauxhurst, J., Krichevsky, M., and Atlas, R. M.: Numerical taxonomic studies on microorganisms isolated from Artic and sub-Arctic marine environments. Proceedings of International Symposium on Microbial Ecology. Springer-Verlag. Heidelberg, 1978.

Kaneko, T., Krichevsky, M. I., and Atlas, R. M.,: Numerical taxonomy of bacteria from the Beaufort Sea. J. Gen. Microbiol. 110:111-125, 1979..

Krichevsky, M. I., The microbial information system (MICRO-IS): A system overview. FDA By-Lines. 9:217-222, 1979.

Walczak, C. A.: The Microbial Information System - Complex Data Analysis. FDA By-Lines. 9:251-253, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 DE 00044-09 ODIR

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Handling of Microbial Strain Information by Computers.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

Micah I. Krichevsky Chief, Microbial Systematics Sec. ODIR NIDR

Leslie L. Love, Biological Laboratory Technician ODIR NIDR

COOPERATING UNITS (if any) Food and Drug Administration, Rockville, MD; American Type Culture Collection, Rockville, MD; Center for diseas Control, Atlanta, GA; Veterans Administration, Long Beach and Los Angeles, CA; Univ. of Louisville, Louisville, KY

LAB/BRANCH

SECTION
Microbial Systematics

INSTITUTE AND LOCATION
NIDR, NIH, Bethesda, MD

TOTAL MANYEARS: 2.2	PROFESSIONAL: 1.5	OTHER: .7

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Microbial strain data are being entered into a data bank which provides such services as: data on specific organisms and/or groups of organisms, a locator service for strains with special characteristics, identification of unknown isolates, cluster analysis definition of parameters of taxa, data management and report writing aids, aids in quality control, and communication of data via common format. Files of primary data on a large number of microorganisms provide a resource for asking both ecological and epidemiological questions of interest in dental research. Programs are being developed and tested to enter, retrieve, and analyze the data in a variety of ways for epidemiological, diagnostic, taxonomic, ecological, etc. uses. The long term goal is to establish a world-wide data bank at a series of cooperating centers. The system originally developed for bacteria is now being expanded to include the algae, yeasts, molds and protozoa.

PHS-6040
(Rev. 10-76)

COOPERATING UNITS: R. Gryder, Food and Drug Administration
F. Benedict, EDRO, Food and Drug Administration
R. Gherna, American Type Culture Collection
D. Brenner, Center for Disease Control
V. Dowell, Center for Disease Control
J. Brooks, Center for Disease Control
L. Wayne, Veterans Administration
V. Sutter, Veterans Administration
R. Atlas, University of Louisville

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 DR 00250-02 ODI

PERIOD COVERED

October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Algorithms for Microbial Systematics

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

Cynthia A. Walczak Computer Programmer ODIR NIDR
Micah I. Krichevsky Research Chemist ODIR NIDR
Paula Mercer Computer Programmer ODIR NIDR

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SECTION

Microbial Systematics

INSTITUTE AND LOCATION

NIDR, NIH, Bethesda, MD

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.9	1.8	0.1

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A feature frequency analysis program has been written which tabulates features for groups of organisms and the uniqueness of each feature for each group. The uniqueness of a feature for organisms from a specific disease may be determine Ranking the features by their mean deviations gives their ability to partition the groups most efficiently. We are developing an algorithm for displaying the level of redundancy of features to aid the microbiologist in choosing the "best" set of features useful in identification by probabilities.

By appropriate redesign of numerical taxonomic programs, their versatility and cost effectiveness have been greatly enhanced. The similarity calculations may be used to develop group statistics using taxonomic and any other logic. This allows such direct comparisons as taxonomically derived groups with groups based on disease state from which isolated and, thus detection of organisms associated with particular disease states.

PHS-6040
(Rev. 10-76)

Annual Report of the Laboratory of Developmental Biology & Anomalies
National Institute of Dental Research

The major efforts in the Laboratory of Developmental Biology and Anomalies are on the prevention and treatment of inherited and acquired defects in oral-facial development. These projects include studies on the development of facial tissues, the possible roles of environmental agents and of genetic factors in oral-facial malformation, connective tissue formation and the interaction of normal and tumor cells with matrix proteins.

Several changes have occurred in personnel. Dr. Hynda Kleinman was promoted to a permanent position. Dr. J. Pennypacker, Z01 DE 00253-02 has left to assume a position in the Department of Zoology, University of Vermont. Dr. S. Rennard has transferred to the NHLBI and Dr. D. Salomon to NCI. Activities will be continued in their research areas by other investigators. A contract to study the distribution of type IV collagen in oral and other tissues with the Department of Pathology, University of Innsbrück has been satisfactorily completed. In May, 1979 the activities in the Craniofacial Development Section were reviewed by outside scientists including representatives of the Scientific Counselors, NIDR. This completes the review of LDBA research activities which began with the review of the Connective Tissue Section in June, 1978.

Connective Tissue Section

Chemotaxis - Major advances have been made in defining the events involved in chemotaxis. The N-formylmethionine peptides discovered to be chemotactic in this Laboratory were found to bind to specific receptors on the surface of macrophages and polymorphonuclear leucocytes. The interaction of the chemoattractants with the receptor has been found to activate enzyme systems which transfer methyl groups from S-adenosyl methionine to proteins and phospholipids. Demethylation reactions follow very rapidly allowing the membranes to return to a quiescent state. Repeated exposure to the attractant or to a gradient of attractant leads to a cyclic reaction and a continual turnover of membrane methyl groups. These methylation reactions alter the permeability and the rigidity of the cell membrane, thus presumeably causing the orientation of cytoskeletal elements and the directed motion of the cell.

Based on the enhanced susceptibility of persons bearing tumors to infections, we have sought a tumor factor that impairs chemotaxis. The chemotactic response of cells obtained from mice bearing a fibrosarcoma was found to be reduced. A small peptide with a molecular weight less than 1000 was purified from the tumor and found to depress phagocyte chemotaxis by inhibiting the methyl transfer reactions referred to above. The structure of the tumor peptide is under study. Since reduced resistance to infection is a major factor in the morbidity of cancer, it is possible that an understanding of this tumor factor will open new approaches to cancer chemotherapy. Further, such peptides may allow modulation of tissue response in inflammatory disorders.

Interaction of Environmental and Genetic Factors in Oral-Facial Malformations - Mycotoxins such as the cytochalasins are widely distributed fungal products known to be able to induce malformations of the neural tube in embryos. We have made a systematic survey among various strains of mice and found that the susceptibility of these strains to the teratogenic effects of cytochalasin D varies widely. Two strains (OEL-N and a^ea^e) were found to be several times more sensitive to cytochalasin D than C57Bl or Swiss-Webster mice. The susceptibility to cytochalasin D was found to be inherited in a recessive pattern. While the molecular basis for this difference is not known and may be different for different strains a similar sensitivity could occur in some humans and relate to the putative role of fungal toxins in human malformations.

Increased susceptibility to the teratogenic effects of vitamin A has been related to a genetic defect. In these studies, we have investigated mutant mice bearing the OEL gene. Mice homozygous for the OEL gene have open eyelids and cleft palate at birth. Mice heterozygous for the OEL gene show low levels of spontaneous malformations but a greater susceptibility to malformations from retinyl palmitate (a form of vitamin A) than their normal littermates. The OEL heterozygote and other normal mice show equal but very high sensitivity to the teratogenic actions of retinoic acid. Studies with L. DeLuca, NCI, indicate that mice with the OEL gene convert retinol to retinoic acid at a very rapid rate when compared to other mice. These studies suggest that a metabolic abnormality in the OEL mice leads to the conversion of an essential dietary factor to a highly teratogenic metabolite.

Cell Attachment - Fibronectin, a large glycoprotein, binds fibroblasts and other cells to collagen. Our previous studies localized the binding site for fibronectin on collagen to a single region of the molecule coincident with the collagenase cleaveage site. Now we have carried out studies of the receptors for fibronectin on the cell surface. These studies show that the oligosaccharides occurring in certain higher gangliosides such as GD_{1A} and GT_1 are able to bind to fibronectin and block the binding of fibronectin to the receptors on the cell surface. This activity is dependent on at least two intact sialic acid residues in the heteropolysaccharide. These studies indicate that the higher gangliosides or proteins bearing similar oliogsaccharides can serve as the cell surface receptors for fibronectin. The reduced binding of fibronectin by transformed cells could be due to the known defect in ganglioside synthesis that accompanies transformation.

Not all connective tissue cells attach to collagen through fibronectin. We have found that chondrocytes attach to collagen by a different attachment protein, which we have named chondronectin. Chondronectin is present in serum and in cartilage and is produced by chondrocytes but not by other cells. Other cells may also produce unique attachment proteins that have a specific affinity for their cell of origin. Such factors may explain the tissue localization of certain cell types.

The attachment properties of metastatic tumor cells from mice have been found to differ from those of normal and nonmetastasizing transformed cells. The metastatic cells produce little or no collagen and fibronectin and bind preferentially to type IV (basement membrane) collagen. Normal cells bind equally well to types IV and I collagen through fibronectin. Since metastatic cells in a population of tumor cells can be selected for by their ability to bind to type IV collagen, a quantitative in vitro assay may be developed to predict metastatic potential.

Basement Membrane Constituents - Three constituents of basement membranes have been isolated from a tumor producing basement membrane. These include type IV collagen, a large glycoprotein (laminin) and a heparan sulfate proteoglycan. Immunological studies suggest that the basement membranes in normal tissues contain similar macromolecules. The three constituents localize to different regions of the basement membrane and probably have different functions. The type IV collagens have a structural role while the proteoglycan probably controls the permeability of the basement membrane. Laminin may regulate cellular interactions.

Antibodies prepared against these basement membrane proteins, as well as other connective tissue proteins, are being used as the basis of a new analytical procedure which requires an enzyme linked to antibody. The method is simple, sensitive and easily adapted to other macromolecules. Using this method, it has been possible to measure mouse and human fibronectin production by hybrid cells and to localize the gene for human fibronectin to chromosome 8.

Craniofacial Development Section

Teratology - An in vitro screening system to detect compounds with teratogenic activity has been developed using differentiating neural crest and limb mesenchyme cells. The addition of known animal and human teratogenic agents such as diphenylhydantoin and excess vitamin A causes alterations in the growth or differentiation of these cells. The screening system is also capable of detecting teratogenic compounds which require metabolic modifications for activity.

The differentiation of limb mesenchyme cells into chondrocytes involves the loss of cell surface fibronectin when the cells become committed to this phenotype. Excess amounts of the teratogen, vitamin A, block this process by enhancing the accumulation of fibronectin at the mesenchymal cell surface, thereby preventing the loss of fibronectin necessary for normal chondrogenesis.

Glucocorticoids are known to cause facial clefting and growth retardation in experimental animals. Previously, we have been able to demonstrate, using various mouse strains, a correlation between the sensitivity to glucocorticoid-induced cleft palate and the levels of glucocorticoid receptors present in the developing craniofacial region. In the human fetus, glucocorticoids are known to cause growth retardation and are suspected of causing facial clefting. We have been able to demonstrate alterations in the glucocorticoid receptors in cultured dermal fibroblasts from persons with a strong familial history of facial clefting. These studies suggest that alterations in glucocorticoid receptors may underlie certain oral facial abnormalities in humans. The information from these studies may be of potential use in genetic counseling for facial clefting.

Cell Differentiation - Peptide growth factors, such as epidermal growth factor (EGF), are know to influence the growth and differentiation of a number of cell types in culture. We have found a fetal growth factor in the mid and late gestation mouse embryo which resembles but is not identical to EGF. This fetal growth factor appears to be involved in the growth and differentiation of epithelial cells in the developing palatal shelves. Our studies have also shown that the newborn mouse is dependent upon EGF in the first few weeks of life. During this time EGF stimulates the proliferation and keratinization of the oral mucosa and epidermis which enhances tooth eruption and eyelid opening. This EGF is apparently obtained through the maternal milk before synthesis begins in the salivary gland at 3 to 4 weeks of age.

The growth and differentiation of most cells and tissues in culture are usually dependent upon the presence of serum whose hormone composition is largely unknown. Others have described media lacking serum in which cells will grow. We have developed a defined culture medium, containing hormones and growth factors such as insulin and EGF which supports the growth and differentiation of mouse teratocarcinoma cells, rat mammary epithelial cells, fetal limb mesenchyme cells and secondary palates. The use of this medium will facilitate defining the hormonal requirements for a number of cell types in culture.

Cranial neural crest cells are totipotent, in that they will differentiate into a number of cell types *in vivo*. Our studies have shown that certain macromolecular components of the serum are responsible for influencing crest cell differentiation. In addition, dimethylsulfoxide (DMSO) has been found to be a reversible inhibitor of crest cell differentiation into pigmented cells, possibly by altering the activity of a serum factor.

The differentiation of the dental papilla mesenchyme cells into dentin secreting odontoblasts is dependent upon an interaction of mesenchyme cells with the adjacent basal lamina, which is of epithelial cell origin. Tunicamycin, a specific inhibitor of protein glycosylation, reversibly inhibits this differentiation and prevents the incorporation of fibronectin into the basal lamina.

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In Vitro. In Press.

THSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DE 00006-19 DB

PERIOD COVERED

October 1, 1978 to September 30, 1979

N01 DE 52477

TITLE OF PROJECT (80 characters or less)

Studies on Leucocyte Chemotaxis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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COOPERATING UNITS (if any) LGCB, PB, and LCS of the NIMH; ERRB of the NICHHD; Dept. of Pharmacology, Medical College of Virginia; Dept. of Pathology, University of Connecticut Health Center; Division of Allergy and Infectious Diseases, University of Washington Medical School and Dept. of Medicine, Boston University

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Laboratory of Developmental Biology & Anomalies

SECTION

Connective Tissue Section

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4.50	3.00	1.50

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES XX (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The ultimate goal of this project is to understand the molecular events as cells migrate along a chemical gradient. Most of our studies utilize leukocytes and small formylmethionyl peptide attractants. Tyrosine appears to be an essential component of the cell surface receptor, and certain hydrophobic peptide esters inhibit agonist-receptor interaction. The events triggered by the interaction of peptide with surface receptor include activation of both a membrane phospholipase and a protein methylesterase, these may contribute to chemotaxis by increasing membrane fluidity and inducing conformational changes in contractile proteins respectively. The cell may terminate its response to formylmethionyl peptides by oxidizing the methionyl sulfur of the attractant. Other regulatory systems under study include the antichemotactic effects of materials derived from tumors as well as glucocorticoids. Initial studies on fibroblast chemotaxis indicate that motility in this cell may involve processes similar to those in the leukocyte.

Introduction - A major aspect of inflammation is the influx of leukocytes, mainly in response to leukoattractants generated at the affected area. We have been studying molecular events of this chemotactic response *in vitro* using as chemoattractants synthetic formylmethionyl peptides which may be related to bacterially-produced attractants. We have previously found that these peptides act at a specific receptor on the leukocyte and that both N-formylation of the methionine and the presence of an aromatic amino acid residue in the terminal position of a tripeptide, are required for maximal activity in a peptide. Nuclear magnetic resonance studies (J. Loiselle, Univ. Conn. Health Center, Farmington, Conn.) have indicated that formylmethionylleucylphenylalanine, a most potent attractant, has a unique conformation in solution which depends upon the formyl group. This may partly explain the high affinity of this peptide for the leukocyte binding site. At present we are examining the initial interaction between attractant and cellular binding site, the nature of the chemical signal transmitted to the motility apparatus to produce directed motion, and the manner in which the chemotactic response is regulated. We have also initiated studies on fibroblast chemotaxis.

Methods - The sources of neutrophils were from rabbit peritoneal exudates and human volunteers. Both assays for chemotaxis *in vitro* using modified Boyden chambers and for peptide-binding to receptors have been described (Schiffmann, E., Showell, H.J., Corcoran, B.A., Ward, P.A., Smith, E., and Becker, E.L. (1975) *J. Immunol.* 114: 1831-1837). Determination of enzymatic activities such as phospholipase and protein methylesterase was performed according to standard procedures (Hirata *et al.*, bibliography for this report; Gagnon, C. (1979) *Biochem. Biophys. Res. Commun.*, in press).

Results - *Interaction between attractant and binding site* - With the aid of a contractor who synthesizes compounds for us we have continued studies on structure-activity relationships of chemotactic peptides. Formylmethionyl-leucyl-phenylalanyl-O-Benzylester (FMet-Leu-Phe-OBz), a less polar derivative of FMet-Leu-Phe, has been found to be 10-fold more active an agonist upon neutrophils and 10,000-fold more active upon macrophages than the parent tripeptide. Replacement of the formyl group with the t-butoxycarbonyl in a series of benzyl esters resulted in the production of weak (10^{-4}M) inhibitors of chemotaxis which were, however, specific in that chemotaxis to complement-derived attractant was less affected than chemotaxis to synthetic peptides or the bacterial factor. These studies could lead to the development of potent antagonists which may be useful in purifying the peptide receptor through affinity labelling techniques. Such compounds might also be effective in regulating *in vivo* chemotaxis.

The receptor appears to contain an essential tyrosine, since treatment with tetranitromethane, a specific reagent for tyrosine, inactivates the binding site. This finding may explain the requirement for a terminal aromatic amino acid residue for maximal activity of N-formylmethionine tripeptides.

The nature of the chemical signal within the cell - In collaboration with Drs. Hirata and Axelrod, NIMH, we have found that chemotactic peptides specifically stimulate the turnover of N-methylated phospholipids in the neutrophil. Attractants enhance phospholipase A_2 activity, resulting in the release of both arachidonic acid and lysolecithin from the cell membrane. These findings are further linked to chemotaxis by the observation that quinacrine, an inhibitor of phospholipase, inhibits both chemotaxis and the release of arachidonic acid from the cell. These events may partly account for the changes in membrane charge and fluidity which accompany chemotaxis. We have also found that peptide chemoattractants specifically stimulate protein methylester turnover, a result similar to that found for bacterial chemotaxis. The reversible esterification of protein carboxyl groups could contribute to conformational changes in proteins required for the directionally motile behavior of the cells.

Preliminary experiments indicate that increased levels of cyclic AMP in the cells increase the activity of phospholipase A_2. Addition of either dibutyryl cyclic AMP or peptide attractant plus theophylline raised phospholipase activity by 50 percent in 10 min. Chemotaxis is partly inhibited (40 to 50 percent) when cyclic AMP accumulates. Our results raise the possibility that cyclic nucleotide-catalyzed phosphorylation of protein may be involved in the phospholipase activation induced by chemoattractants. Such a mechanism may contribute to the regulation of chemotaxis.

The regulation of chemotaxis - We have been studying the inhibitory action of material derived from tumors since it has been found by others that cells from tumor-bearing animals show depressed chemotaxis. Ethanol extracts from a rapidly proliferating mouse fibrosarcoma were found to contain a material which inhibits chemotaxis in both neutrophils and macrophages. The material was not toxic to the cells. Peritoneal macrophages from mice bearing the fibrosarcoma showed depressed chemotaxis compared to cells from normal mice. We have determined some properties of the tumor factor. Gel filtration studies indicated that the molecular weight of the material was less than 1,000. The anti-chemotactic activity was destroyed by proteases, suggesting it was a peptide. Partial purification of the material by liquid and paper chromatography resulted in two compounds which contained relatively high amounts of isoleucine, leucine and glutamic acid. This material does not compete with attractant for the receptor, but reduces the incorporation of labelled methyl groups from (^3H methyl) methionine into phospholipids of the cell. It, therefore, appears to interfere with transmission of the chemotactic signal generated by the activated receptor. Complete identification of this tumor-produced inhibitor and elucidation of its mode of action obviously would be of great significance in explaining how tumors subvert host surveillance systems.

Another mechanism for regulating chemotaxis may be through metabolism of the chemoattractants. We have previously showed that formylated peptides were hydrolyzed by the leukocytes. An alternative metabolic pathway appears to be an oxidative one. We have now found, in collaboration with Dr. R.A. Clark, Boston University Medical Center, that a purified myeloperoxidase system derived from neutrophils, inactivated methionine-containing attractants such as C5a and formylated methionyl peptides. The inactivation resulted from oxidation of the thioether of methionine to sulfoxide/sulfone. Peptide attractants not containing methionine were not affected. Similar results were obtained with intact neutrophils in the presence of attractants and may signify an important regulatory mechanism for the cell in controlling its own chemotactic response.

Glucocorticoids are anti-inflammatory agents whose mechanism of action has been of broad interest. Others have shown that these compounds inhibit lysosomal enzyme release from leukocytes and reduce the chemotactic response. We have now found that a 16-hour preincubation of neutrophils with pharmacological levels (1 μm) of a glucocorticoid, fluocinolone, protects the cells against the degranulating effects of a subsequent treatment with phorbol esters. The latter compound at 10 nM causes vigorous exocytosis and markedly depresses both chemotaxis and peptide binding in cells not treated with steroid. Since phorbol esters are inflammatory stimuli, a study of the counteracting effects of steroids observed here may yield some leads on their mechanism of action and on post-receptor events in chemotaxis. For example, α phorbol esters are known to enhance phospholipase activity. We plan to determine if the steroids promote the synthesis of a lipase inhibitor.

Applications of formylated peptides to clinical problems - It is conceivable that systemic or local administration of these peptides could be used to control appearance of leukocytes at certain sites in the host. Such manipulation might be of benefit in combatting infections or ameliorating allergic inflammations. In collaboration with L. Altman, University of Washington Medical Center, we have carried out studies on the survival of thermally injured mice. The purpose is to determine whether peptide attractants could significantly enhance the survival rate of such animals which were subsequently exposed to lethal doses of Pseudomonas. Utilizing a conjugate of an FMet peptide and sulfadiazine and mixtures of this antimicrobial agent and peptide attractant, we treated mice as described above, dressed their wounds with these compounds, and observed their survival rates.

Preliminary indications are that mixtures of antimicrobial agent and attractant significantly protect the mice. This might be applied in treatment of burn victims, in whom infection is a major risk.

Fibroblast chemotaxis

Others have shown that fibroblasts respond chemotactically to lymphocyte-derived factors that might be expected to be formed as a consequence of inflammation. We have initiated a study on fibroblast chemotaxis since such behavior may be important in wound repair and since our findings on leukocyte motility might be applied here. Dr. V.G. Muller has developed an assay for fibroblast chemotaxis utilizing techniques originated by A. Postlethwaite. The requirement for collagen as an attachment substrate has been confirmed, but its claimed role as a true attractant remains unclear. Fibronectin (FN), protein involved in the attachment of fibroblasts, has been shown to be a true attractant. A mixture of gangliosides inhibited chemotaxis to both FN and a lymphokine specific for fibroblasts. This indicates that reversible adherence of the cell is essential to chemotaxis and does not depend upon the nature of the attractant. Inhibition of methylation and phospholipase activities depressed chemotaxis. The attractants were found to change the turnover of both protein methyl esters and methylated phospholipids, events which are also prominent in neutrophil chemotaxis.

Proposed course of project

We plan to continue structure-activity studies on peptides in collaboration with contractors, Drs. R. Freer and E.L. Becker (N01-DE-52477). In studies with the neutrophil various blocking groups on the amino group of methionine in tripeptide-O-benzyl esters will be prepared and tested as inhibitors of chemotaxis. Using another approach, trifluoromethyl methionine will be incorporated into a tripeptide and the latter tested for biological activity. We also plan to study the macrophage peptide receptor. For this purpose a new high affinity ligand, FMet-Leu-Phe-NBz, is being prepared as a radiolabelled compound.

We intend to pursue investigations on the molecular events during transmission of the chemical signal triggered by the attractant. The results from glucocorticoid treatment of cells suggest that a phospholipase inhibitor is increased, leading to diminished membrane turnover. We plan to study the nature of (protein) and the generation of such an inhibitor (whether dependent upon de novo synthesis, or its degradation inhibited by steroid). We also will determine whether there is an inhibitor for the methylesterase enzyme in neutrophils. The cyclic nucleotide studies bear on these points, and we shall attempt to find out whether cyclic AMP enhances phospholipase via protein phosphorylation.

We plan to identify the tumor-derived inhibitor and investigate more fully its mechanism of action. Oxidative mechanisms will also receive our attention. We plan to determine whether myeloperoxidase treatment of receptor-bearing cellular fragments will inactivate the binding site. This may well occur since intact sulfhydryl groups were previously shown to be required for maximal binding of agonist to receptor.

Studies with possible clinical applications of the peptides will be continued. It is planned to synthesize other conjugates of chemoattractant peptides and antimicrobial agents and then to test them for efficacy in promoting resistance to infection in burned animal models. We shall also study the metabolism of these conjugates (e.g. hydrolysis) in both leukocytes and bacteria.

We intend to continue studies on motility in non-phagocytic cells such as fibroblasts. The role of phospholipid metabolism will be more extensively studied (existence of inhibitors of phospholipase, effect by levels of cyclic AMP upon this enzymatic activity in the cell). We also intend to examine fibroblast chemotaxis in cells from patients with abnormalities in wound healing such as diabetes.

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U.S. DEPARTMENT OF
HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DE 00009-18 DB

PERIOD COVERED

October 1, 1978 to September 30, 1979

N01 DE 82410

TITLE OF PROJECT (80 characters or less)

Chemistry and Biosynthesis of Connective Tissue

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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Univ. of Pittsburgh

LAB/BRANCH

Laboratory of Developmental Biology & Anomalies

SECTION

Connective Tissue Section

INSTITUTE AND LOCATION

NIDR, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 8.25	PROFESSIONAL: 5.75	OTHER: 2.50

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this project is to study the formation, function and destruction of connective tissue components in normal and diseased states. Particular attention is directed toward collagen and proteoglycan. Current aspects of this project include (1) characterization of the matrix components in a tumor which produces basement membrane, (2) the role of collagen in diseases and (3) interaction of tumor cells with collagens.

PHS-6040
(Rev. 10-76)

Extracellular matrices composed of various collagens, proteoglycans and other macromolecules create and maintain tissue form. We have found that alterations in the synthesis or processing of these extracellular matrix components underly various heritable disease of connective tissue such as osteogenesis imperfecta, the Ehlers-Danlos syndrome etc. primarily because the tensile strength of collagen fibers is lessened. Since a variety of distinct collagens have been identified and are associated only with certain cells, mechanisms for the sorting out of specific cells into tissues have been elucidated (see for example Z01 DE 00230-03 DB).

We are examining the ways in which tumor cells interact with collagen matrices. Highly metastatic cells preferentially attach to type IV (basement membrane) collagen over other collagens (types I, II, III, and V). It may be possible to predict the metastatic potential of tumor cells based on their ability to bind to type IV collagen and to degrade this protein.

We are using a tumor model to study the molecular structure of basement membranes. Three unique constitutents of this and other basement membranes have been isolated including type IV collagens, laminin and basement membrane proteoglycan. Since basememt membranes are the first extracellular matrices to arise in the embryo, they may have unique roles in development. Clearly, they are also involved in a number of common disorders including diabetes, bullous pemphigoid and the spread of tumors. Further studies will attempt to identify the abnormalities in such disorders.

Components of Basement Membranes - Since basement membranes are minute anatomical structures, they have not been easy to characterize. For this reason, we have been studying the basement membrane which is made in large amounts by a murine tumor. Our strategy has been to isolate a component of the tumor matrix, prepare specific antibody against this material and use the antibody to establish the occurrence and distribution of the isolated macromolecule in normal tissues. So far we have prepared three unique constitutents of basement membranes, two of which (laminin and basement membrane proteoglycan) have not previously been described in any tissue.

Basement Membrane Collagens - Previous studies by others suggested that basement membranes contained a genetically distinct collagen but its structure was not characterized. In defining the basement membrane collagen, we have found that the tumor matrix as well as authentic basement membranes contain the same two distinct collagen molecules, type IV-1 and type IV-2. These proteins are synthesized as procollagen precursor forms which are processed to lower molecular weight macromolecules. The type IV molecules are larger than other collagens and differ in their susceptibility to various enzymes.

Tryggvason, Robey and Martin

Laminin, A Basement Membrane Glycoprotein - We have isolated a large glycoprotein, laminin, from the tumor which is also a major constituent of normal basement membranes. Laminin has a molecular weight of about a million daltons and contains chains of 200,000 and 400,000 daltons. Laminin is not codistributed in the basement membrane with Type IV collagen since laminin is found in the lamina lucida and type IV collagens in the lamina densa.

Martin, Robey, Foidart, Rennard with Timpl, Max Planck Institute

Basement Membrane Proteoglycans - Recently, with Dr. John Hassell, NEI, we have isolated a proteoglycan, BMI, from the tumor that appears to be localized only in basement membranes. BMI contains about 30% protein and 70% heparan sulfate. Antibody prepared against BMI reacts with both the tumor matrix and tissue basement membranes. It is likely that BMI because of its negatively charged groups has a functional role in the basement membrane in restricting the passage of acidic macromolecules.

Martin, Robey, Barrach, Wilzcek, Rennard with Hassell, NEI

Function of Basement Membrane Components - The role of the basement membrane components will be tested by administering specific antibodies to fetal animals. So far we have found that antibody to type IV collagen causes kidney and lung damage while antibody to laminin has no effect. The role of the proteoglycan will be investigated in a similar fashion.

Foidart, Yaar, Brown

Tumor Cells - Certain metastatic tumor cells produce a collagenase that degrades basement membrane but not other collagens. These metastatic cells, in contrast to normal cells and nonmetastatic tumor cells, attach preferentially to type IV over types I, II, III, and V collagens. The number of metastases obtained in animals is increased by injecting the cells that bind to type IV collagen. These observations suggest that the metastatic cells represent only a portion of the cells in the tumor. We are developing a metastatic index for tumor cells based on their ability to bind to types IV and I collagens and produce the type IV collagenase.

Murray and Martin with Liotta NCI

The Ehlers-Danlos Type VI Syndrome - Collagen from a patient with the Ehlers-Danlos type VI syndrome has been examined. This patient has been found to accumulate a collagen containing a pNα2 chain. This indicates that the cleaveage of the amino terminal peptides from the pro α1 chains occurs but not from the pro α2. The patient's cells contain normal amounts of procollagen N-protease. Purified chick procollagen N-protease cleaved the amino terminal peptides from the pro α1 but not from the pro α2 chains from the patient's collagen. These data indicate an abnormal resistance of the pro α2 chain to procollagen N-protease presumeably due to an amino acid substitution in the pro α2 chain.

Martin, Steinman with McKusick, Johns Hopkins

Fibronectin - New immunoassays have been established for measuring connective tissue proteins. These have been used to measure fibronectin production by hybrid cells and localize the human fibronectin gene to chromosome 8. Further, it has been found that the reduced binding of fibronectin by transformed cells is greatly increased by dexamethasone. Presumeably, dexamethasone increases the number of cell surface receptors for fibronectin.

Rennard, Foidart with Furcht, U. of Minnesota, with Church, U. of Pittsburgh

Significance - Many disorders alter normal connective tissue development as well as function. In our studies, the function of various types of collagen is being investigated. These studies show that these collagens can be used as molecular markers to identify specific cell types. Specific antibodies have been prepared to these collagens and used in histological and pathological studies. Defects in one or another collagen are associated with certain diseases and developmental disorders.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER
		Z01 DE 00024-13 DB

PERIOD COVERED

October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Developmental Processes in Genetically Controlled Malformations

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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COOPERATING UNITS (if any) Dr. W. Lena Austin, Dept. of Microbiology, Howard University Washington, D.C.; Dr. Robert F. Cranley, Dept. of Pathology, St. Agnes Hospital, Baltimore, Maryland; Dr. Luigi De Luca, NCI; Dr. David Newsome, NEI; Dr. Beverly White, NIAMDD.

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Laboratory of Developmental Biology & Anomalies

SECTION

Connective Tissue Section

INSTITUTE AND LOCATION

NIDR, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
6.95	1.95	5.00

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES XX (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The objectives of this project are to describe the genetic mechanisms and developmental processes in mutant and highly inbred animals with hereditary predisposition to congenital malformations, particularly involving the face and limbs, and to utilize these animals as experimental systems for the study of the processes of congenital malformation. Mouse strains with hereditary malformations as Mendelian or non-Mendelian traits have been discovered in this laboratory or are obtained from others. Comparison of normal and abnormal development are carried out in embryos of timed gestational age using gross examination of living embryos, whole mount preparations and histological sections as well as biochemical analyses of tissues and of cultured organs and cells. Genetic analysis involves segregation analysis, selection of sublines, cross breeding of abnormal and normal lines and genetic linkage studies. Agents such as drugs, vitamins and hormones are used as probes both for the study of gene action in hereditary malformations and for the study of possible human teratogens.

PHS-6040
(Rev. 10-76)

Objectives

The objective is to determine the biochemical basis of genetic differences in malformations. Inherited differences in mice of different genotypes are being used to detect the biochemical mechanisms of birth defects. Mutants with generalized defects of growth resulting in facial clefts and dwarfism are being explored in detail at the biochemical level. Mutants with differing sensitivity to teratogenic agents are being used as probes for the cellular and biochemical basis of abnormal development.

Methods

Selected stocks of mice carrying mutations producing malformations and highly uniform inbred laboratory mice differing in metabolic responses are our test systems. Timed pregnancy and genetic uniformity permit specific studies of critical periods of development and the specific effect of gene action on morphogenesis. Timed treatments with appropriate drugs, hormones or growth factors act as detailed probes of the biochemistry of the developmental process.

Findings

The findings will be discussed for several of the genetic types of mice being investigated.

Open eyelid with cleft palate oel

The mouse strain OELS has the recessive gene oel which produces the lethal malformation of open eye lid with cleft palate. It differs from OELN, a closely related normal strain, in its differential sensitivity to teratogenesis induced by retinol. Both strains are highly and equally sensitive to retinoic acid teratogenesis. We have been working to delineate the mechanism of the differential sensitivity.

Transplacental passage studies utilizing triticated retinoic acid and retinol indicate that the drugs cross the placenta in both OELS and OELN mice. Cytosol binding receptors for retinol in the maxillary processes of newborn mice were the same for OELS and OELN. However, we have found in collaboration with Dr. L. De Luca, NCI, that the sensitive strain metabolizes retinol much more rapidly than the resistant strain. Further these studies indicate that the sensitive animals convert the retinol to retinoic acid to which both strains are more sensitive. These studies suggest that the malformations are due to an enhanced conversion of dietary retinol to the more teratogenic retinoic acid. The effect of lowering dietary retinol levels on the incidence of malformations is under study.

Disproportionate micromelis Dmm

In homozygous state Dmm has been found to have severe disorganization of the structure of the epiphysis and cleft palate with shortening and flattening of the snout so that the tongue protrudes. Studies with Drs. Pennypacker and Kleinman have demonstrated reduction of both collagen and proteoglycans in the epiphyseal cartilage. The histological and biochemical defects are much less severe in heterozygotes. These observations suggest that Dmm may be a structural gene and that there may be a chromosome defect large enough to detect. Cells from heterozygous animals have been established in culture and are being studied for possible major chromosome abnormality by Dr. Beverly White of NIAMDD. The present data suggest that the synthesis of type II collagen in Dmm/Dmm is abnormal and that the proteoglycan reduction is secondary. Further studies of the collagen and proteoglycan defects in cultured cells of the Dmm/Dmm, Dmm/+ and +/+ genotype are planned.

Cartilage matrix deficiency cmd

The homozygous newborn cmd/cmd resembles Dmm/Dmm. However, the histological and biochemical patterns are different. Collaborative studies with Drs. Kimata and Pennypacker have demonstrated that the cmd/cmd epiphyseal cartilage lacks the cartilage specific proteoglycan while there is no abnormality of collagen. The reduced proteoglycan content is associated with a reduced water content of the cartilage and reduced extracellular matrix volume.

The complete recessive pattern of cmd suggests that it may be a regulatory gene rather than a structural gene coding for the proteoglycan. Similar reasoning applies to cn, Achondrophasia which has a similar type but in which the biochemical defect is not yet identified. Experiments crossing these two mutants with Dmm are planned to evaluate possible genetic and biochemical interaction.

Brachymorphic bm

Several experiments have been carried out to further specify the nature of the defective sulfation in the bm/bm animal. Drs. Pennypacker and Kimata in collaborative studies have found that this deficiency occurs in several tissues although it is of greater magnitude in cartilage than, for example, in skin. Day 14 embryo bm/bm have higher cyclic AMP levels than normal embryos. This may be secondary to the PAPS defeciency or to a separate but genetically linked trait.

Recurrent epilation Er

The homozygous gene Er results in fusion of epithelial surfaces. The oral structures are fused to each other and the limbs and tail are fused to each other and to the trunk resulting in a pupoid fetus which cannot breathe at birth. This defect starts after day 12 and is associated with intrauterine bleeding. Steroid treatments have failed to modify the expression of the trait. Attempts to culture the skin cells have not yet succeeded. No abnormalities of pemphigus and pemphigoid antigens have been detected in collaborative studies with Dr. John Stanley of NCI. No abnormality of surfactant precursor uptake or keratinzation has been found as yet.

Forelimb defect fld

This recessive mutant has a generalized defect of bone that resembles some of the types of osteogenesis imperfecta with fractures and campomelia observed in the newborn. The affected animals are born alive but rarely survive to weaning. Histological studies have not been diagnostic and preliminary studies of bone collagen are in progress. Alizarin preparations show fractures of bones in the limbs and ribs.

Teratogenic responses of inbred strains

Genetic differences in responses to teratogenic alkaloids which are known to occur in foods and elsewhere in the environment might provide a process resulting in the observed irregular distribution of human birth defects. To test the teratogenicity of some mycotoxins and possible genetic differences we have been collaborating with Dr. Lena Austin of Howard University in a study of the cytochalasins. These substances produce a characteristic neural tube defect when administered at the end of the first week of gestation. Significant differences among strains and a recessive genetic pattern for susceptibility to teratogenic response to cytochalasin D has been found. Cytochalasin E is more embryo lethal but less teratogenic than D while B is an order of magnitude less toxic than either.

Future plans

The metabolic defect in the OELS mouse that results in sensitivity to vitamin A will be characterized specifically. We will first test the hypothesis that retinoic acid is formed as a result of a block in normal retinol metabolism. The effect of vitamin A free diet on the expression of oel will also be studied. Similar extentions of biochemical studies on the defect in the other mutants are planned. In addition the animals with defects will be used to test interactions with teratogens, and among different types of genes producing defects. New mutants will be examined for the presence of biochemical defects producing malformation or dwarfism.

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Brown, K.S.: Implications of Receptor Physiology for Teratogenesis. In Bixler, D. and Melnick, M. (Eds.). Academic Press, New York, In press, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)

U.S. DEPARTMENT OF
HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DE 00025-13 DB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Regulation of Collagen Biosynthesis in Normal and Diseased Tissues

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Larry M. Paglia	Staff Fellow	NIDR	DB
OTHER:	George R. Martin	Chief, LDBA	NIDR	DB
	Lucille A. Ouellette	Biologist	NIDR	DB
	Joseph Wilczek	Biologist	NIDR	DB

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Developmental Biology & Anomalies

SECTION
Connective Tissue Section

INSTITUTE AND LOCATION
NIDR, NIH, Bethesda, Maryland

TOTAL MANYEARS: 3.50	PROFESSIONAL: 1.25	OTHER: 2.25

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☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this project is to investigate the regulation of collagen biosynthesis in normal and diseased cells. Here we are concerned with translational control of biosynthesis and posttranslational steps in helix formation.

PHS-6040
(Rev. 10-76)

The purpose of this project is to investigate the regulation of collagen biosynthesis in normal and disease states. We are concerned both with the regulation of collagen type and the amount of collagen produced. Our approach involves use of a cell-free protein synthesizing system to examine the steps in collagen mRNA translation and posttranslational modification.

The type and amount of collagen present in a tissue is closely regulated, with changes in either parameter associated with embryonic development and with disease states. The type of collagen is probably determined by selective gene transcription, however, the cells synthesizing collagen must have some mechanism for determining the amount of collagen present in the tissue and adjusting its synthesis.

Recent evidence suggests that the amino terminal extension peptide of procollagen, normally cleaved during processing of the molecule, may act as a feedback inhibitor of its synthesis. We have investigated the possibility that this peptide acts at the translational level in a reticulocyte lysate cell-free system. The lysate efficiently translates mRNA from collagen-producing tissues into procollagen. When Type I NH_2-terminal peptide was added to the translation reaction, pro $\alpha1(I)$ and pro $\alpha2$ synthesis was inhibited. This decrease was determined by specific immunoprecipitation and by gel electrophoresis of the cell-free products. The synthesis of both chains was equally reduced. Translation of the mRNA for other proteins was unaffected. The analogous peptide from Type III procollagen was also inhibitory while peptides derived from other regions of the collagen molecule were not.

RNA from chick sternum and rat chondrosarcoma has also been successfully translated into Type II procollagen. Addition of Type I propeptide to these reactions inhibits Type II procollagen synthesis, while, Type III propeptide does not affect translation of Type II mRNA. We are currently investigating the mechanism and specificity of this propeptide inhibition.

Procollagen synthesized in the cell-free system is also being analyzed with regard to its ability to form a native triple helix. RNA coding for Type I and Type II procollagen was translated and the resulting protein tested for helicity by resistance to pepsin digestion. Our data indicate at least partial helix formation even without hydroxylation or glycosylation of the molecule. Cell-free synthesized procollagen could also be precipitated with ammonium sulfate at 20% saturation, a property of native but not denatured collagen. Thus, it appears that the assembly of the triple helical procollagen molecule is a process governed by its polypeptide chains. That is, the amino acid sequence of the chain is sufficient to establish the tertiary structure of the protein.

Significance: Feedback inhibition of mRNA translation may be a means by which cells regulate collagen synthesis. Changes in disease states may represent defects at this level as well as at the level of transcription. Investigations of procollagen helix formation provide valuable information on the ability of this protein to self-assemble *in vivo* and provide new insights into the role of posttranslational modifications to the protein.

PUBLICATIONS:

Paglia, L., Wilczek, J., Diaz de Leon, L., Martin, G.R., Horlein, D. and Muller, P.: Inhibition of procollagen cell-free synthesis by amino terminal extension peptides. *Biochemistry*. In press, 1979.

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Breitkreutz, D., Diaz de Leon, L., Paglia, L., Gay, S., Swarm, R. and Stern, R.: Histological and biochemical studies of a transplantable rat chondrosarcoma. *Cancer Research*. In press, 1979.

Diaz de Leon, L., Breitkreutz, D., Paglia, L., Zeichner, M. and Stern, R.: Partial characterization of procollagen messenger ribonucleic acid in a murine chondrosarcoma. *Connective Tissue Research*. In press, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DE 00230-03 DB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Attachment of Cells to Collagen

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Hynda K. Kleinman	Research Chemist	NIDR	DB
OTHER:	A. Tyl Hewitt	Postdoctoral Fellow	NIDR	DB
	George R. Martin	Chief, LDBA	NIDR	DB
	J. Clifford Murray	Visiting Fellow	NIDR	DB
	Charlotte M. Wilkes	Biologist	NIDR	DB

COOPERATING UNITS (if any) Dept. of Human Biological Chemistry & Genetics, The University of Texas Medical Branch, Galveston, TX; NCI; NINCDS; Dept. of Medicine, University of Wisconsin, Madison, WI; and University of San Francisco Medical School, San Francisco, CA

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Laboratory of Developmental Biology & Anomalies

SECTION
Connective Tissue Section

INSTITUTE AND LOCATION
NIDR, NIH, Bethesda, Maryland

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.20	2.45	1.75

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☐ (a) HUMAN SUBJECTS XX(b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this project is to study the mechanism of cell attachment to collagenous matrices. Particular attention is directed towards (1) the attachment properties of various cultured and non cultured cells and whether cells synthesize or utilize attachment proteins different than fibronectin, (2) the nature of the attachment protein(s) specificity for different collagens and reconstituted collagen and (3) the interaction of adhesion proteins with other molecules and with cells.

Introductions and Objectives - Many cells exist surrounded by a collagenous matrix. The role of collagen in the tissue is probably more than structural since collagen matrices promote the growth and differentiation of cells. Fibroblastic cells do not bind directly to collagen but are linked to the collagen by a large glycoprotein, fibronectin. We are investigating the role of collagen and fibronectin in cell attachment. Our studies indicate that a variety of collagen attaching proteins exist including different ones for fibroblasts, chondrocytes and epidermal cells.

Methods - The interaction of cells with collagen is studied *in vitro*. Cells are obtained either as cell lines or from a variety of tissues and separated by standard methods. Human skin fibroblasts and CHO cells were obtained from American Type Culture Collection, chondrocytes from chick embryonic sternum and a rat chondrosarcoma, hepatocytes from adult rat liver and epidermal cells from guinea pig epidermis.

Attachment properties of cultured cells - The interaction of cells with the collagen-attachment protein complex is likely to play a major role in determining the distribution of cell types in tissues. A variety of cells are being tested for their attachment properties, i.e. rate, serum dependence and collagen specificity. Cultured fibroblastic cells such as human skin fibroblasts, CHO, 3T3, show enhanced attachment to collagen and require serum fibronectin for attachment. Cells such as hepatocytes derived directly from tissue also show serum dependence while epidermal cells do not. Epidermal cells probably synthesize their own attachment protein because (1) they attach very slowly, (2) their attachment is not enhanced by fibronectin, (3) they do not make fibronectin, (4) they adhere specifically to type IV collagen, and (5) they differentiate into multilayered squamous epithelial type cells on type IV collagen.

Murray, Wilkes and Kleinman

Chondrocytes - The involvement of fibronectin in the interaction between chondrocytes and collagen in cartilagenous matrix is unlikely since immunofluorescence studies indicate that fibronectin is absent from cartilage. For this reason, the attachment of chondrocytes to collagen is being investigated. These cells appear to attach to collagen by means of a factor other than fibronectin. We have named this factor chondronectin. Initial studies on the specificity of embryonic chick chondrocyte sternal attachment to collagen showed that, in the presence of serum, chondrocytes attached best to type II collagen substrates (60% attachment), although they also attached to type I (47%), III (45%), and IV (30%) collagens. This is in contrast to CHO cells which bound equally well to all four types in the presence of serum (Kleinman *et al.*, 1978, *J. Invest. Dermatol.* 71:

9-11). When fibronectin is removed from serum by passage over a collagen-sepharose column, the chondrocyte attachment enhancing activity of serum is retained while that for CHO cells is lost. Purified fibronectin promoted the attachment of CHO cells as effectively as serum but had no effect on chondrocyte attachment. Fractionation of serum using Amicon filtration and molecular sieve column chromatography indicate that the chondrocyte-attachment factor has a molecular weight greater than 3×10^5. The chondrocyte factor can be separated from fibronectin in serum by means of DEAE-cellulose column chromatography. It elutes as a single peak of activity at a lower ionic strength than fibronectin. These data confirm that a high molecular weight factor other than fibronectin is responsible for the attachment of chondrocytes to collagen.

Hewitt and Kleinman

Chondrosarcoma cells - Chondrocytes from a rat chondrosarcoma have also been studied for attachment properties. These cells adhere best to type II collagen over the other genetically distinct collagens. This preference is enhanced when the dishes used for the attachment assays are coated with partially purified type II collagen, which contains proteoglycans and other glycoproteins. β-xyloside inhibits the attachment of these cells. This again suggests that proteoglycans may be involved in the attachment of chondrosarcoma chondrocytes.

Kimata and Hewitt

Interaction of fibronectin with collagen - The nature of the fibronectin-collagen interaction is being explored. The interaction of fibronectin with native collagen is weak and thus its physiological role in cell adhesion is unclear. In addition, Grinnell has reported that some fibroblasts can efficiently adhere to native collagen substrates in the absence of serum. We find that cells require fibronectin for adhesion to native collagen substrates. However, in the absence of fibronectin cells will adhere to native collagen substrates if 5×10^{-3} M phosphate or greater is present. The phosphate acts by binding to the collagen and will support cell adhesion to Ascaris collagen, which does not bind fibronectin. Phosphate appears to interact more efficiently with native collagen. This effect on adhesion is not physiological since the cells cannot be released from the substrate by trypsin.

The direct interaction of fibronectin with native collagen is also being examined. Using reconstituted collagen fibers, we find that fibronectin does bind to native collagen. This interaction can be blocked by denatured collagen fragments which contain the specific fibronectin binding site.

The ability of fibronectin to crosslink to collagen via blood factor $XIII_a$ (transglutaminase) is also being explored with regard to specificity of collagen substrates. Ascaris collagen cannot participate in this reaction while native collagen, denatured collagen α chains and certain cyanogen bromide fragments of the $\alpha1(I)$ collagen can. The interaction is specific in that $\alpha1(I)$-CB7 and $\alpha1(I)$-CB8 will crosslink while $\alpha1(I)$-CB6 will not.

Wilkes and Kleinman

Ganglioside inhibition of fibronectin-mediated adhesion - Gangliosides are cell surface glycolipids which are present in the lipid layer of the cell membrane via their ceramide portion. We found that gangliosides inhibit fibronectin-mediated cell adhesion. Only the complex gangliosides GD_{1a} and GT_1 were active while GM_1, GM_2 and GM_3 were inactive. The sugar portion of the molecule contains the activity since ceramides (the lipid) alone were inactive. The sugar portion alone was active and oxidation of the sialic acid residues resulted in loss of activity. The gangliosides appear to be acting by binding to the portion of the fibronectin molecule which binds to the cell surface. Cell adhesion is blocked if the fibronectin is pretreated with the ganglioside but not blocked if the collagen is pretreated. The inhibition will also occur after fibronectin has bound to collagen. In addition, immunologic studies indicate that the ganglioside can directly bind to fibronectin. Preincubation of the cells with gangliosides has no effect. Thus, gangliosides specifically inhibit fibronectin-mediated cell-adhesion by binding to fibronectin through their sugar residues. These results suggest that certain gangliosides may act as the cell surface receptor for fibronectin.

Wilkes and Kleinman

Significance - Various genetically distinct collagens exist in association with a specific type of cell including chondrocytes with type II collagen and epidermal cells with type IV collagen. Such cells produce proteins that mediate their attachment. Our studies explain the molecular basis of this sorting out and the manner in which tissues are formed from the interaction of cells with the extracellular macromolecules they produce.

PUBLICATIONS:

Kleinman, H.K., Murray, J.C., McGoodwin, E.B. and Martin, G.R.: Connective tissue structure: The attachment of cells to collagen. J. Invest. Dermatol. 71: 9-11, 1978.

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Kleinman, H.K., Hewitt, A.T., Murray, J.C., Liotta, L.A., Rennard, S.I., Pennypacker, J.P., McGoodwin, E.B., Martin, G.R. and Fishman, P.H.: Cellular and metabolic specificity in the interaction of adhesion proteins with collagen and with cells. J. Supramol. Struct. In press, 1979.

Kleinman, H.K., Martin, G.R. and Fishman, P.H.: Ganglioside inhibition of fibronectin-mediated cell adhesion to collagen. Proc. Natl. Acad. Sci. USA. In press, 1979.

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Hassell, J.R., Pennypacker, J.P., Kleinman, H.K., Pratt, R.M. and Yamada, K.M.: Enhanced cellular fibronectin accumulation in chondrocytes treated with vitamin A. Cell 17: 821-826, 1979.

Hewitt, A.T., Kleinman, H.K., Pennypacker, J.P. and Martin, G.R.: Identification of an adhesion factor for chondrocytes. Proc. Natl. Acad. Sci. USA. In press, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 DE 00253-02 DB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Development of Cartilage

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	John P. Pennypacker	Senior Staff Fellow	NIDR	DB
OTHER:	A. Tyl Hewitt	Postdoctoral Fellow	NIDR	DB
	Koji Kimata	Visiting Associate	NIDR	DB
	Willard A. Lee	Chemist	NIDR	DB
	Hugh Varner	Postdoctoral Fellow	NIDR	DB

COOPERATING UNITS (if any)

None

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Laboratory of Developmental Biology & Anomalies

SECTION
Connective Tissue Section

INSTITUTE AND LOCATION
NIDR, NIH, Bethesda, Maryland

TOTAL MANYEARS: 4.75	PROFESSIONAL: 2.75	OTHER: 2.00

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES XX (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The factors influencing chondrogenesis are under study. Particular attention is being directed towards the extracellular matrix of developing cartilage and its relationship to normal cellular function.

In these investigations, use is being made of limb mesenchyme cell cultures, mature chondrocytes in culture, cartilage producing tumors and cartilage from various animal mutants which may serve as models for human chondrodystrophies.

PHS-6040
(Rev. 10-76)

Studies on Chondrogenic Expression in vitro

I. Embryonic limb mesenchyme cells plated at high density differetiate into cartilage within four days. However, at low density chondrogenesis does not occur. The responsiveness of limb mesenchyme to various growth factors was tested in an attempt to enhance proliferation and obtain chondrogenesis from low density cultures. Ornithine decarboxylase (ODC) activity was utilized as a biochemical marker for biological response to the factors. The developing limb bud in vivo has a rise in ODC activity prior to chondrogenesis, and this is also true when mesenchymal cells are plated at high density and allowed to undergo chondrogenesis in culture. However, at low density the ODC activity remained low and the mesenchyme did not differentiate. The mesenchymal cells were found to be unresponsive to fibroblast growth factor, FGF at both high and low density. However, they responded to epidermal growth factor, EGF, but at low density only. The EGF effect did not enhance chondrogenesis. Work is continuing to determine if the matrix helps dictate EGF responsiveness by the mesenchymal cells.

Pennypacker

II. Previous studies have shown that a high molecular weight fraction of embryo extract inhibits chondrogenic expression. The purpose of this study is to develop an assay procedure for embryo extract activity and to utilize it to follow the purification of the active component of embryo extract. A 180,000 MW protein is now being characterized as a marker for chondrogenic expression. Preliminary evidence suggests this protein is Type II procollagen and its appearance is rapidly (< 24 hr) affected by embryo extract. Studies are now in progress to determine the nature of the effect on the putative procollagen.

Pennypacker and Varner

Chondrocyte Interaction with Collagen

Recent studies indicate that chondrocyte interaction with collagen is mediated by a serum protein, designated chondronectin, whereas fibroblasts bind via fibronectin. The purpose of this study was to determine whether alterations in chondrogenic expression are correlated with changes in the requirements for attachment to collagen. Vitamin A was used to induce a fibroblast-like phenotype in cultured chondrocytes. Preliminary results suggest that changes in phenotype are correlated with altered attachment requirements.

Hewitt and Pennypacker

Animal Models for Human Chondrodystrophies

The cartilage of a new mouse mutant (cartilage matrix deficiency) was analyzed by biochemical and immunofluorescent techniques. This mutant is characterized by disproportionate dwarfism and cleft palate. The studies indicate that the cartilage-specific proteoglycan is absent in the extracellular matrix and that the primary defect involves synthesis of the proteoglycan core protein.

Pennypacker and Kimata

PUBLICATIONS:

Binderman, I., Greene, R.M. and Pennypacker, J.P.: Control of calcification of differentiating skeletal mesenchyme *in vitro*. *Science* In press, 1979.

Hassell, J.R., Pennypacker, J.P., Kleinman, H.K., Pratt, R.M. and Yamada, K.M.: Enhanced cellular fibronectin accumulation in chondrocytes treated with vitamin A. *Cell* 17: 821-826, 1979.

Hewitt, A.T., Kleinman, H.K., Pennypacker, J.P. and Martin, G.R.: Identification of an adhesion factor for chondrocytes. *Proc. Natl. Acad. Sci. USA*. In press, 1979.

Kleinman, H.K., Hewitt, A.T., Murray, J.C., Liotta, L.A., Rennard, S.I., Pennypacker, J.P., McGoodwin, E.B., Martin, G.R. and Fishman, P.H.: Cellular and metabolic specificity in the interaction of adhesion proteins with collagen and with cells. *J. Supramol. Struct.* In press, 1979.

Pennypacker, J.P. and Goetinck, P.F.: Reversible inhibition of chondrogenic expression by certain hyaluronidase preparations. *J. Embryol. Exp. Morphol.* In press, 1979.

Pennypacker, J.P., Hassell, J.R., Yamada, K.M. and Pratt, R.M.: The influence of an adhesive cell surface protein on the chondrogenic expression *in vitro*. *Exp. Cell Res.* 121: 411-415, 1979.

Pennypacker, J.P., Wilk, A.L. and Martin, G.R.: Differentiation of mesenchyme cells into chondrocytes in culture: Effect of teratogens. In Neubert, D., Merker, H.J., Nau, H., and Langman, J. (Eds.): *Role of Pharmacokinetics in Prenatal and Perinatal Toxicology*. Stuttgart, Georg Thieme, pp. 411-419, 1978.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 DE 00275-01 DB

PERIOD COVERED

October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Biological Testing of Fluoride

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	George R. Martin	Chief, LDBA	NIDR	DB
OTHER:	Kenneth S. Brown	Medical Director	NIDR	DB

COOPERATING UNITS (if any) University of Minnesota; Litton Bionetics; NCI; LB

LAB/BRANCH

Laboratory of Developmental Biology & Anomalies

SECTION

Connective Tissue Section

INSTITUTE AND LOCATION

NIDR, NIH, Bethesda, Maryland

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.10	.10	0.00

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this project is to study the action of fluoride on various systems used to detect clastogenic or mutagenic substances. To date fluoride has been examined in microbiological assays used to detect mutagens and found to be non mutagenic. No effects on chromosome structure were noted in animals given widely different levels of fluoride. DNA repair after X-ray was unchange by fluoride. No genetic effects of fluoride were noted in a recessive lethal test of fluoride on drosophila. The data indicate that fluoride has no mutagenic activity.

PHS-6040
(Rev. 10-76)

Fluoride has been widely used to suppress caries. At the levels recommended (0.5-1 ppm), no detrimental effects of fluoride on the health of individuals has been noted. At very high fluoride intakes, alterations in bone metabolism can occur and are associated with degenerative changes in bone.

It is the purpose of this project to examine fluoride in some of the more recently developed tests which detect mutagenic or clastogenic activity. Considerable epidemiological data indicate that fluoride consumption does not increase the incidence of cancer or birth defects. The tests described below provide corraborative experimental information.

In our initial studies, we measured the numbers of abnormal chromosomes in mice from colonies raised on very different levels of fluoride. Bone levels of fluoride differed some 450 fold. The level of chromosomal abnormalities in bone marrow and testes cells were low in both groups and not significantly different. The rate of sister chromatid exchange was similar in animals from the two groups. Mice give low (1 ppm) to high levels (100 ppm) of fluoride had similar levels of chromosomal abnormalities which did not differ from the level of abnormalities present in animals receiving no fluoride suppliment.

Slight and not statistically significant increases in XY chromosomal dissociation were noted in the range 2-4% in animals supplimented with fluoride. Great variation in this parameter (up to 40%) have been noted in previously published studies and the XY chromosome dissociation is believed to arise during preparation of the chromosomes for examination.

No effect was noted of fluoride levels in a recessive lethal test which detects genetic effects of mutagenic substances.

Fluoride was not found to be mutagenic when tested in microbial assays (the Ames Test).

Taken together the data show no genetic or mutagenic actions of fluoride.

PUBLICATIONS:

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF** **INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 DE 00149-05 DB

PERIOD COVERED
October 1, 1978 to September 30, 1979 — N01 DE 52452

TITLE OF PROJECT (80 characters or less)
Cellular Proliferation and Differentiation During Craniofacial Development

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Robert M. Pratt, Jr.	Research Chemist	NIDR	DB
OTHER:	Frances B. Cannon	Bio Lab Tech (Micro)	NIDR	DB
	Elizabeth A. Horigan	Research Biologist	NIDR	DB
	Webster C. Leyshon	Biologist	NIDR	DB
	General L. Mosley, Jr.	Bio Lab Tech (Animal)	NIDR	DB
	David S. Salomon	Senior Staff Fellow	NIDR	DB
	Michael Silver	Postdoctoral Fellow	NIDR	DB
	Russell Sim	Visiting Associate	NIDR	DB
	Irma P. Thesleff	Visiting Associate	NIDR	DB

COOPERATING UNITS (if any)
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LAB/BRANCH
Laboratory of Developmental Biology & Anomalies

SECTION
Craniofacial Development Section

INSTITUTE AND LOCATION
NIDR, NIH, Bethesda, Maryland

TOTAL MANYEARS: 9.25	PROFESSIONAL: 4.25	OTHER: 5.00

CHECK APPROPRIATE BOX(ES)
☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The long range purpose of this project is to understand the regulation of cellular proliferation and differentiation in craniofacial tissues. At present we are (1) developing an in vitro method for screening teratogens (2) developing methods to culture craniofacial tissues; (3) determining the biochemical events that regulate palatal development; and (4) determining the biochemical events in odontoblast differentiation and incisor tooth eruption.

I. Teratogen Screening

Animal tests used for determining the possible teratogenic activity of compounds is both time consuming and expensive. We have developed a rapid *in vitro* screening assay for teratogens using differentiating neural crest cells (also see Z01 DE 00257-01 DB Greenberg) and limb mesenchyme cells. Teratogenic compounds inhibit the growth and/or differentiation of one or both cell types in contrast to the non-teratogens tested so far. The teratogenic effect is dose dependent and can be quantitated by measuring the amount of a differentiated cell product (i.e. melanin or proteoglycan). The conversion of non-teratogenic compounds, such as cyclophosphamide, to teratogenic metabolites is achieved with liver microsomes incorporated into the cultures. Future studies (previously Z01 DE 00138-04 DB) will be directed toward developing other cell types which can complement the present system and to develop serum-free culture media in order to facilitate teratogen testing *in vitro*.

Horigan (also see Greenberg Z01 DE 00257-01 DB)

II. Palatal Development

Clefts of the primary and secondary palate are common and serious birth defect of interest to the NIDR. Normal palatal development involves growth, elevation and fusion of the palatal processes. An alteration in any one of these processes could lead to cleft lip and palate.

The distribution of the adhesive glycoprotein fibronectin is restricted to the mesenchymal cell surface and extracellular matrix of the primary and secondary palate. It appears to be involved in cell to cell and cell to matrix interactions. Fibronectin is not found on opposing palatal epithelia and therefore is not involved in epithelial cell adhesion. Future studies are aimed at identifying the glycoproteins which are involved in this cell to cell interaction in palatal development.

Silver

Previous studies have demonstrated a correlation, using various mouse strains, between the sensitivity to glucocorticoid-induced cleft palate and the levels of glucocorticoid receptors present in the developing facial region. In the human fetus, glucocorticoids are known to cause growth retardation and is suspected of causing facial clefting. In preliminary experiments, we have found altered glucocorticoid receptors in dermal fibroblasts from persons with a strong family history of facial clefting. These results suggest that an hormonal dysfunction may be associated with facial clefting.

Salomon, Leyshon

III. Terminal Differentiation and Growth Factors - Terminal differentiation of the palatal medial-edge epithelial cells is determined early in palatal development. This process can be precociously stimulated in vitro by cyclic AMP analogs or prevented by epidermal growth factor (EGF). Our studies on isolated or recombined palatal epithelium have shown that EGF prevents cell death only when the epithelium is in combination with its mesenchyme. These results suggest that the mesenchyme may provide a necessary macromolecular substrate (collagen, fibronectin) which enables the epithelial cells to respond to the mitogenic effects of EGF. EGF along with glucocorticoids may play a role in normal palatal epithelial cell growth and differentiation. This is supported by our recent studies demonstrating the presence of an EGF-like growth factor and its receptor in the mid and late gestation mouse fetus.

Sim, Salomon, Leyshon

The growth of most cells and tissues, such as the palate, in culture is dependent upon the presence of serum, although the components in serum are largely unknown. We have been able to develop a completely defined medium containing growth peptides such as insulin and EGF which will support the growth and differentiation of mouse teratocarcinoma cells, mammary epithelial cells, embryonic limb mesenchyme cells and secondary palates in organ culture. Future studies will be directed toward understanding the hormonal requirements for various cells and tissues in culture.

Salomon, Horigan

IV. Tooth and Limb Development

The differentiation of the dental papilla mesenchyme cells into odontoblasts is thought to be dependent upon an interaction with the adjacent basal lamina which is of epithelial origin. Tunicamycin, a specific inhibitor of protein glycosylation, reversibly blocks this differentiation of mesenchymal cells into odontoblasts. Tunicamycin exerts its effect by altering the biochemical components esp. fibronectin and the ultrastructure of the basal lamina, thereby disrupting epithelial - mesenchymal interactions. Future studies are aimed at characterization of the components of the basal lamina necessary for this interaction and to determine the biochemical nature of cell surface macromolecules on the mesenchymal cells which are important for this specific interaction.

Thesleff, Horigan

The teratogen vitamin A inhibits limb chondrogenesis both in vivo and in vitro. Previous studies of ours suggested that vitamin A exerts its effect by altering fibronectin. Recent studies have shown that vitamin A prevents the loss of fibronectin from the cell surface of mesenchymal cells which have become committed to chondrogenesis by enhancing the affinity of the cell surface for fibronectin. Future studies (previously Z01 DE 00135-04 DB) are directed toward understanding the role that fibronectin plays in limb mesenchyme cell to cell and cell to matrix interaction both in vivo and in vitro.

Silver, Leyshon

The eruption of the incisor teeth in the newborn mouse can be precociously stimulated by EGF. We have found that this effect is specific for EGF in that a variety of other growth peptides do not elicit this effect. EGF appears to exert its effect by increasing the proliferation and keratinization of the oral mucosa. Future studies are aimed at identifying the cells in the oral mucosa which are responsive to EGF during this neonatal period.

Sim, Leyshon, Thesleff

PUBLICATIONS:

Diewert, V. M. and Pratt, R. M.: Selective inhibition of mandibular growth and induction of cleft palate by diazo-oxo-norleucine (DON) in the rat. Teratology. In press, 1979.

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Greene, R. M. and Pratt, R. M.: Correlation between cyclic AMP levels and cytochemical localization of adenylate cyclase during development of the secondary palate. J. Histochem. Cytochem. 27: 924-931, 1979.

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Hassell, J. R., Pennypacker, J. P., Kleinman, H. K., Pratt, R. M. and Yamada, K. M.: Enhanced cellular fibronectin accumulation in chondrocytes treated with vitamin A. Cell 17: 821-826, 1979.

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Pratt, R. M., Wilk, A. L., Horigan, E. A., Greenberg, J. H. and Martin, G. R.: Screening for teratogens in vitro. In Bixler, D., Melnick, M. (Eds.): Academic Press. New York, In press 1979.

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Salomon, D. S. and Pratt, R. M.: Inhibition of growth in vitro by glucocorticoids in mouse embryonic facial mesenchyme cells. J. Cellular Physiology. 97: 315-328, 1978.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 DE 00257-01 DB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Differentiation of Cranial Neural Crest Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Judith H. Greenberg	Senior Staff Fellow	NIDR	DB
OTHER:	Elizabeth A. Horigan	Research Biologist	NIDR	DB
	Robert M. Pratt, Jr.	Research Chemist	NIDR	DB

COOPERATING UNITS (if any)

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Craniofacial Development Section

INSTITUTE AND LOCATION
NIDR, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 3.00	PROFESSIONAL: 1.25	OTHER: 1.75

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☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Cranial neural crest cells, which contribute to the formation of many craniofacial structures, differentiate in culture into two of their normal derivatives, neurons and pigment cells. As an approach toward defining the factors which affect normal and abnormal neural crest development, we are examining various aspects of their differentiation in culture. The well-defined and reproducible differentiation of neural crest cultures has enabled us to develop a system to screen for teratogens in vitro.

I. Factors Affecting Differentiation of Neural Crest Cells in Culture

Chick cranial neural crest cells cultured in medium containing horse serum (HS) differentiate into cells resembling neurons and contain choline acetyltransferase (CAT) activity, whereas those cultured in fetal calf serum (FCS) or calf serum differentiate into melanocytes. Up until the third day of culture, the pathway of differentiation of the neural crest cells can be changed by shifting the type of serum in the medium.

Dimethyl sulfoxide (DMSO) prevents the pigmentation of neural crest cells without affecting their growth rate or their differentiation into neuron-like cells. The inhibition of pigmentation is reversible and involves a late step in melanogenesis, since cells maintained in the presence of DMSO contain premelanosomes. Increased concentrations of FCS or of a high molecular weight fraction of FCS enhances pigmentation in control cultures and partially overcomes inhibition of pigmentation in DMSO-treated cultures. These results suggest that DMSO may alter the activity of a factor in FCS thereby preventing it from interacting with neural crest cells to promote pigmentation. Experiments are in progress to define the mechanism of action of DMSO.

Greenberg

II. Collagen Synthesis by Cultured Neural Crest Cells

Several distinct tissue - specific collagens exist in the embryo. Their synthesis changes during development and may play a role in cell differentiation and tissue morphogenesis. Undifferentiated neural crest cells, as well as pigmented and neuronal cultures, synthesize types I and III collagen. When cells differentiate into melanocytes, the ratio of collagen to non-collagenous protein does not change, but when they differentiate into neuronal cultures the ratio of collagen to non-collagenous protein is markedly reduced. These results suggest that the relative amounts of collagen reflect the pathway of differentiation of the cells and may provide a useful criterion by which to assess normal and abnormal differentiation of these cells.

Greenberg

III. Neural Crest Cultures as a Screening System for Teratogenic Compounds

Replicate cultures of neural crest cells differentiating into neuronal and pigmented cells were subjected to treatment with a variety of drugs known to be teratogenic in animals and with several drugs which have no documented teratogenic effect. We have observed a relation between teratogenicity *in vivo* and the ability of a compound to alter the growth, morphology, or differentiation of these cells. Drugs which require metabolic activation to produce their effect can be tested in cultures by combining the drug with the microsomal fraction of rat liver. Analogous studies have been performed using limb mesenchyme cells which differentiate into chondrocytes (see Z01 DE 00149-04 DB). The results of these studies were comparable and suggest that these differentiating cells may provide a system to screen for teratogenic agents and to elucidate the mechanism of action of certain teratogens.

Pratt and Horigan

Future Plans

Future studies will further characterize the factors that are involved in the differentiation of neural crest cells and relate them to possible inductive or permissive factors *in vivo*. These approaches include (1) developing a completely defined culture medium for maintaining undifferentiated neural crest cells; (2) fractionating serum and testing components for their effects in modulating neural crest differentiation in otherwise defined medium; and (3) purifying serum factors which contain inducing activity and preparing antibodies against these factors in order to localize by immunofluorescence possible inductive interactions *in vivo*.

Studies on teratogens in culture will also continue, with an emphasis on the mechanism of action of certain teratogens and on quantitating the effects of teratogens on neural crest cultures. In addition, we will try to establish cell lines derived from neural crest cells which will make it possible to grow larger numbers of cells for investigations at the biochemical level.

PUBLICATIONS:

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Pratt, R. M., Wilk, A. L., Horigan, E. A., Greenberg, J. H. and Martin, G. R.: Screening for teratogens in vitro. In Bixler, D., Melnick, M. (Eds.): *Academic Press*. New York, In press 1979.

Wilk, A. L., Greenberg, J. H., Horigan, E. A., Pratt, R. M. and Martin, G. R.: Detection of teratogenic compounds using differentiating embryonic cells in culture. In Vitro. In press.

ANNUAL REPORT OF THE LABORATORY OF BIOCHEMISTRY
NATIONAL INSTITUTE OF DENTAL RESEARCH

Laboratory programs have remained nearly constant in both size and content for the past several years. The creation of the Proteoglycan Chemistry Section this past year recognized the already present independence and importance of that program. The Protein Chemistry Section and the Enzyme Chemistry Section are the other two subdivisions of the Laboratory. The number of persons in the Laboratory is about 25, the exact number varying as visitors and persons in postdoctoral-type positions arrive and leave. The ratio of research to research-support staff is about 2:1. This ratio has increased in recent years as the number of job positions has decreased but has been made possible by a greater reliance on instruments and disposable supplies. The ratio of persons in rotating postdoctoral-type positions and more senior visitors to tenure research staff is also about 2:1. This ratio reflects a major element of training in Laboratory programs.

The major interest in the Enzyme Chemistry Section is the family of enzymes called transglutaminases. All catalyze the same reaction, the formation of an amide bond between a peptide-bound glutamine residue and an amine donor. The amine can be any of a variety of low molecular weight compounds or the ε-amino group of a peptide-bound lysine. In the latter case a crosslink, an $\varepsilon(\gamma$-glutamyl)lysine bond, is formed between proteins or polypeptides. This method of stabilization is well established for fibrin and there is evidence for the crosslink in cell membranes, myofibrils, seminal plasma proteins and hair proteins. Recent studies in the Section have shown that another type of crosslink can be formed by the enzyme utilizing a diamine or polyamine to bridge two peptide-bound glutamine residues. Since polyamines such as spermidine and spermine and the diamine putrescine are important biological constituents, this means of making crosslinks or of modifying proteins may be important in certain physiological process.

In related studies in the Enzyme Chemistry Section, transglutaminases have been found in megakaryocytes and endometrium of uterus and in the membranes of several cell types. This membrane-bound enzyme may be a new and specific transglutaminase.

Transglutaminase can be used as a means of artifically introducing crosslinks into proteins. This procedure is useful in determining the relative position of two proteins in larger biological structures. To aid in this approach, bifunctional amines containing a chemically labile bond have been synthesized. After forming a crosslink and isolating the crosslinked complex, the bond can be cleaved to release the constituents and allow their characterization.

Proteoglycans have a protein core and a variety of different kinds of polysaccharide side chains. In cartilage and certain other tissues, proteoglycan exists as a very stable complex with hyaluronic acid, a very long single-chain polysaccharide, and link protein, a small glycoprotein.

The efforts of the Proteoglycan Chemistry Section are devoted to studying the structure, biosynthesis and biological role of these important connective tissue components. The systems currently in use are cartilage cells from a rat chondrosarcoma, chick limb bud cells and corneal stroma. Structural studies on cartilage proteoglycan have progressed to the point where specific fragments can be obtained that are suitable for detailed studies including amino acid sequencing. It has been found that in addition to the well-characterized glycosaminoglycan side chains, proteoglycan from chick limb bud cells in culture and from the rat chondrosarcoma contains mannose-rich oligosaccharides similar to those present in glycoproteins. Biosynthetic studies have elucidated the relationship between the synthesis of proteoglycan, hyaluronic acid and link protein. It was found that synthesis in serum-free culture is stimulated by somatomedin and to an even greater extent by insulin. These results are important both for the purpose of providing better defined conditions for growing cells in culture and also for providing a biological system for studying the role of these and other important factors that can be easily manipulated.

Proteoglycan from cartilage can be considered a prototype, but it is by no means the only kind of proteoglycan. Typical of the potential variety of types is the finding of two quite different proteoglycans in corneal stroma explants. One has a single chondroitin sulfate chain and a few oligosaccharides per molecule and the other has one or two keratan sulfate chains and a few oligosaccharides, while cartilage proteoglycan may have more than 100 condroitin sulfate and keratan sulfate chains per molecule.

Emphasis in the Protein Chemistry Section is on collagen structure. Molecular structure, chemistry and biosynthesis are quite well understood. Questions now being asked pertain to high level structure and interactions. The tools being used include electron microscopy, quasielastic light scattering, nuclear magnetic resonance and computer modelling. The picture of the native collagen fibril that is emerging is that there is a high degree of axial order but that there is rapid lateral movement through several or perhaps many conformational states. There is, however, a statistically prefered relationship, and three dimensional order is present. What that order is, is still a matter of controversy. The widely-held 5-stranded microfibril model has been questioned and will at least need some revision.

The assembly of collagen molecules to native fibrils in a well-defined in vitro system is a means of obtaining evidence about structure that may be relevant to in vivo assembly. Studies done in the Protein Chemistry Section have shown that assembly is a multistep process. Long thin filaments that could be 5-stranded microfibrils are intermediates. Their structure and that of even earlier intermediates is being studied by light scattering and by electron microscopy. Procedures for low beam damage electron microscopy and for computer analysis of electron micrographs, which are now nearly in place, are expected to aid these studies. In addition, preliminary studies using the scanning transmission electron microscopes at Johns Hopkins and at Brookhaven are promising.

An important element in the Laboratory programs described above is the interaction between groups in the different Sections. This occurs both at the discussion level and in joint projects. For example, collagen-proteoglycan interactions during collagen assembly are being studied; nuclear magnetic resonance studies in proteoglycan structure *in vitro* have been done; and the role of transglutaminase in wound healing and its possible interaction with collagen is of interest. All of the Laboratory programs also have active collaborative aspects with programs in other parts of NIDR and NIH and with various laboratories around the world.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 DE-00002-29 LB

PERIOD COVERED
October 1, 1978 - September 30, 1979

TITLE OF PROJECT (80 characters or less)

Structural Studies on Collagen

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI :	B. L. Trus	Senior Staff Fellow	NIDR LB
OTHER:	K. A. Piez	Chief	NIDR LB

COOPERATING UNITS (if any)
R. Feldmann, C. Bacon, T. Porter, J. Deleo, W. Barrett, DCRT: A. Steven, M. Navia, V. Nikodem, NIAMD: M. Elzinga, Brookhaven National Laboratory, Upton, New York.

LAB/BRANCH
Biochemistry

SECTION
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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.75	1.50	0.25

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The primary goal of this project is an understanding of collagen structure from the molecular to the fibril level. Emphasis is presently on two main aspects of the general problem. 1) Computer enhancement and reconstruction of electron micrographs to reveal detail not obvious to the eye. 2) Three-dimensional computer models to study and illustrate structural features of collagen and molecular aggregates. Each of the above two approaches has led to the development of computer software that is being applied to other problems in protein structure and electron microscopy. A third aspect of this project, to relate amino acid sequence of collagen to higher level structure, has been inactive this year.

PHS-6040
(Rev. 10-76)

1. Project Description

Objectives:

The primary objective of this project is to determine the structure of the collagen molecule and of aggregates of collagen including the native collagen fibril. Several approaches are being taken. 1) Computer analysis (enhancement and reconstruction) of electron micrographs of collagen aggregates to reveal details not obvious to the eye and to provide quantitative data. 2) Computer modeling of collagen molecules and aggregates to illustrate and test proposed structures. 3) Analysis of collagen amino acid sequences to search for features related to higher level structure. A secondary objective developed during the current year is to extend the computer modeling programs developed for collagen to myosin, a collaborative project with Dr. M. Elzinga, Brookhaven National Laboratory. This is possible because both myosin and collagen are coiled coils, although the helical parameters are very different.

Methods Employed:

Analysis of electron micrographs uses standard enhancement techniques, as developed at the University of Basel and adapted to the computer hardware at NIH, and procedures developed in this laboratory specially suited to our problem. Specifically, electron micrographs are digitized on a Perkin Elmer 1010G microdensitometer. Relevant information is extracted using a Frame Buffer display, and analysis is done using a system of computer programs called PIC. PIC, which is an extension of the Basel system, can call over 100 operations including fast Fourier transforms, Fourier filtering by high and/or low pass filtering, Fourier filtering by masking, calculation and display of the effective optical diffraction, inverse Fourier, analysis of periodic structure, and generation of hypothetical or model electron micrographs. The results of the enhancement and reconstruction are visible immediately on the cathode ray tube of the Frame Buffer and can be recorded on film by a Camera Station. Molecular modeling programs for collagen and myosin were developed using an Evans and Southerland Picture System II interfaced with a DEC 11/70 computer, Frame Buffer, and Camera Station. Knobs are used to change variables such as pitch, supercoil, and angles. Switches allow the selection of a number of viewing options. A keyboard is used to input the one letter amino acid code for display of specific types of amino acids. Since the modeling is essentially a real time operation on the Picture System, it is extremely easy to study different regions of collagen with a variety of starting parameters.

Major Findings:

Development of the PIC system software for electron micrograph enhancement and reconstruction has been a major effort this year. The system is now being used as more and better electron micrographs of collagen are becoming available. The system is also being used by other investigators at NIH with similar research problems.

The molecular modeling programs have been used to produce a thirteen minute color movie of collagen structure. The movie starts by illustrating molecular structure, shows the relationship between two molecules, and concludes by depicting the 5-stranded microfibril which is believed to be a substructure of the native fibril.

The myosin program has been used to study the pitch of myosin along the 148 residues in the available sequence. The pitch can be changed dynamically along the partial sequence to accomodate systematic local variations.

To resolve a conflict between a recently proposed model of the collagen fiber based on x-ray diffraction evidence and the microfibril model supported by other data, we have reinterpreted the x-ray diffraction data. We have proposed a revised model which contains "compressed" microfibrils placing molecules on a pseudohexagonal lattice. The unit cell provides an accurate fit of the observed reflections on the x-ray diffraction pattern and is consistent with other data.

Significance:

Collagen is the major protein of connective tissue and is found in various forms throughout the body. Through interactions with other macromolecules such as proteoglycans, mineral, and cells, it plays an important role in many biological processes during development and in pathological states. These studies on collagen structure are important to understanding these interactions.

Proposed Course:

Approximately 90% of the PIC system has been implemented and tested. While some continuing development of new software can be expected, substantially more emphasis will be on use rather than development. Initial enhancement investigations will be concerned with the early stages of fibril formation, and attempts to identify the structures present. These early aggregates will be prepared as part of another project. Additional applications will be sought for the PIC system, as data aquisition is easier with installation and successful operation of a high resolution microdensitometer. Continuing use of PIC will involve cooperation with A. Steven on virus structure, M. Navia on immunoglobulin structure, and V. Nikodem on two-dimensional gel quantitative analysis.

The real time collagen display system will be kept accurate to latest proposed structure, and can be used to help evaluate more detailed descriptions of collagen. While initial work was limited to backbone atoms, more accurate models are being built to include all the atoms in a particular section.

While analysis of collagen sequences for features related to higher level structure was not pursued this year, we expect to return to this project when new sequence data becomes available.

Additional portions of the myosin sequence will soon be available. The next large segment is in a different portion of the helical part, and will provide an interesting comparison between the two segments.

2. Publications

Piez, K. A. and Trus, B. L.: Sequence Regularities and Packing of Collagen Molecules. J. Mol. Biol. 122:419-432, 1978.

Trus, B. L. and Piez, K. A.: Collagen Structure. A computer drawn, 13-minute color movie.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 DE-00049-08 LB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Physiological Role and Metabolism of Transglutaminases

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI :	S. I. Chung	Research Chemist	NIDR, LB

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Dr. Hee Sik Sun, NIAMDD
Dr. Soo Young Lee, Catholic Medical School, Seoul, Korea

LAB/BRANCH
Biochemistry

SECTION
Protein Chemistry

INSTITUTE AND LOCATION
NIDR, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 1.00	PROFESSIONAL: 1.00	OTHER: 0

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The physiological function and the mode of regulation of the transglutaminases are being studied. The roles of individual transglutaminases in the formation of cellular membranes, in membrane-mediated stimulation of specific cellular function, and in the regulation of tissue matrix stabilization following injury are under investigation.

PHS-6040
(Rev. 10-76)

Objectives

The final phases of protein-tissue matrix (structural proteins, phospholipids, etc.) interaction in the "Cascade Scheme" of hemostasis lead to fibrin formation induced by multi-steps of limited proteolysis. Fibrin monomer spontaneously interacts in a hydrophobic environment to form polymer through an ordered process. Fibrils are formed which show unique 260 Å spacing as characterized by electron microscopy. The stabilization of these fibrils involves enzyme catalyzed ε-(γ-glutamyl)-lysine cross-linking at designated sites between γ-γ chain and α-α chains. A variety of primary amines may interfere with cross-link formation by competing with amino groups of peptide-bound lysine residues.

The transglutaminases catalyze the incorporation of primary amines at the carboxamide group of protein- and peptide-bound glutamine residues and promote formation of ε-(γ-glutamyl)lysine bonds within and between protein molecules. These active enzymes are formed: 1) by a limited proteolysis in plasma during hemostasis and by an association with Ca++ ions, 2) by influx of Ca++ ions into cellular sites from various stimulations that occur in vivo (tissue transglutaminase), 3) by a ligand- or mitogen-induced activation of cells (membrane-bound transglutaminase). These ε-(γ-glutamyl)lysine crosslinks have been found in numerous stabilized proteins of animal tissue.

Also these cross-links may be involved in the stabilization of tissue matrices which provide substraum for selective adhesion of cells and subsequent cell proliferation (i.e., wound healing).

The mode of transglutaminase activation in the ligand-induced interaction of receptor and cytoskeletal component at the cortex of cell and subsequent endocytosis of ligand-receptor complexes are currently under investigation (i.e., receptor-mediated endocytosis).

Extended studies of the various transglutaminases are underway with special emphasis toward physiological function and its mode of regulation.

Major Findings

In continuation of studies carried out in the previous years, investigation of the biosynthesis of protransglutaminase in other types of cell systems is underway.

Using fluorescent antibody techniques, megakaryocytes and endometrium of uterus were found to contain protransglutaminase but not protransglutaminase-binding globulin. In this sense, they are unique and

College, Seoul, Korea. Two separate methods were adopted for this study. 1) Factor XIII deficiency was induced in rabbits by intravenous infusion of IgG isolated from goat anti-rabbit platelet factor XIII, and the deficient state was maintained by daily infusion. Control rabbits were given normal goat IgG. After production of standard skin wounds, the progress of epithelialization, fibroblast proliferation, and collagenous matrix formation was monitored by histological and immunohistochemical methods. In normal rabbits, epithelialization was complete by day 6; fibroblast proliferation by day 10; maximal collagenous matrix accmulation was observed on day 14. In factor XIII-deficient animals, epithelialization was not completed until day 14; fibroblast proliferation and collagenous matrix accumulation were retarded beyond day 20. These observations suggest that factor XIII is involved in early phases of wound healing. 2) In an initial attempt to study the mode of factor XIII in tissue repair, the effect of factor XIII on the growth of fibroblast was examined in vitro. However, the addition of factor XIII to the culture media showed no effect on the growth of cells.

Membrane-bound transglutaminase

Sensitive and specific methods have been developed to detect and quantitate trace amounts of transglutaminases present in the isolated cells. Using peroxidase-conjugated antibody and monospecific anti-transglutaminase, the electron microscopy of hepatocytes and lymphocytes showed clustering of transglutaminase in Golgi regions and endoplasmic reticulum and very light distribution in the inner cell-membrane area.

Evidence has been accumulated for the existence of a completely different type of transglutaminase associated with the membrane-rich particulate fraction of cells. Isolated membrane-rich particulate fraction from rat chondrosarcoma cells showed high specific enzymic activity. This membrane bound transglutaminase activity was totally extractable with detergents. Activity was increased several fold by a limited proteolysis with concomitant change of molecular weight from 100,000 to 50,000. Several fold increase of transglutaminase activity in the rat chondrosarcoma cells and mouse embryo cells is observed upon treatment of cells with nmole quantities of proteases. These increases in enzyme activity are closely associated with ligand-receptor induced clustering and subsequent endocytosis. A survey of plasma cells, as well as structural cells, indicated that almost all of the cells possess two types of transglutaminase, one which is identical with the tissue enzyme and another that is membrane-bound.

Significance

A comprehensive study of various transglutaminases has shown that the enzymes are widely distributed in various cells, in both the cytosol distinct from previously studied hepatocyte systems where both proenzyme and its binding globulin were synthesized and released. Endometrium of uterus plays an important function in furnishing nutrient medium and serves as a matrix for fertilized ovum implantation. Although the biochemical processes involved in ovum implantation are not clearly understood, the possible involvement of covalent cross-links has been postulated. This is based on clinical observation where a high incidence of abortion is observed in congenital factor XIII deficiency.

This implantation system *in vitro*, may serve to be an effective model system to examine the early phases of cell adhesion and adaptation of matrix conditions for cell proliferation.

A method was devised for obtaining intact uterine epithelial cells. This was accomplished by digestion of the sterile everted uteri horns with a mixture of crude trypsin and collagenase. Modified Ham's F-12 culture media was employed containing 0.5% calf serum and six growth factors: insulin, hydrocortisone, transferrin, GHL, somatostatin and thyrotropin. Effects of other hormones, cyclic AMP and diethyl stilbestrol on propagation were negative. Fibronectin was extremely effective in promoting attachment of the cells. Clonization of epithelial cells and myoblasts of uterus has been achieved and each cell has been maintained under the described culture condition for up to 6 months.

Lamprey Fibrinogen

The structure of lamprey fibrinogen has been the object of prolonged investigation because of the unusually large molecular weight of its α-chain, and its unique subunit composition. Limited cross-linking (only γ-γ cross-linking) occurs during the stabilization of lamprey fibrin. Extensive studies on the isolated chains of lamprey fibrinogen by sedimentation equilibrium and gel permeation in both chaotropic solvent and physiological buffer show molecular weights of 110,000, 78,000 and 50,000 for (A) α, (B) β, and γ chains, respectively. If the conventional formulation $(A(\alpha)_2, B(\beta)_2, \gamma_2)$ is used to calculate the molecular weight, the results give a mass of 470,000 far in excess of 360,000 obtained by us. However, the data are in close agreement to a $(A(\alpha), B(\beta)_2, \gamma_2)$ formulation based upon the estimated value of molecular weight. Preliminary studies on CNBr-fragmented α-chain show 11 peptides out of a possible 14 per 110,000 molecular weight in favor of the proposed formulation.

The Role of Factor XIII in Wound Healing

Investigation of the role of factor XIII in wound healing has been initiated in collaboration with Dr. Soo Young Lee of Catholic Medical and the membranes, and suggests that all may be involved in the formation of ε-(γ-glutamyl)lysine crosslinks. These crosslinks are essential for maintaining the permanent rigid structure of many protein molecules. The findings of transglutaminase activity associated with cell membranes and its enhancement by membrane stimulators suggest an additional role in cell membrane protein interaction; i.e., in receptor-mediated endocytosis.

The delayed epithelialization and collagen formation in experimentally-induced factor XIIIdeficient animals, the rapid healing of wounds in factor XIII-treated animals, and enhanced growth of fibroblast on stabilized fibrin plates support previous suggestions of a vital role of factor XIII in wound healing and tissue repair.

Proposed Course

1. Attention will be focused on the possible in vivo interaction between fibrinogen and collagen as catalyzed by transglutaminases and the possible relationships of cross-link formation to cell adhesion and proliferation in wound healing.

2. Studies on the biosynthesis, release, and catabolism of platelet factor XIII will be carried out using guinea pig megakaryocytes. Regulation of factor XIII in rabbit uterus epithelial cells and its role in ovum implantation is under investigation.

3. The mode of activation and involvement of membrane-bound transglutaminase in receptor-mediated endocytosis will be investigated using lymphocytes and fibroblast cell lines.

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U.S. DEPARTMENT OF
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NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 DE-00134-05 LB

PERIOD COVERED
October 1, 1978 - September 30, 1979 N01-DE-72403

TITLE OF PROJECT (80 characters or less)

Structure and Biosynthesis of Proteoglycans

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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Others	:	S.M. De Luca	Senior Staff Fellow	NIDR LB
		J.H. Kimura	Staff Fellow	NIDR LB
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		L.S. Lohmander	Visiting Assoc.	NIDR LB
		R.M. Mason	Visiting Scientist	NIDR LB
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COOPERATING UNITS (if any) Arnold Caplan, Case Western Reserve University Contract (N-01-DE-72403), John Hassell, NEI, Bo Nilsson, NCI, Robin Poole, Shriners' Children's Hosp., Montreal, Peter Fietzek, Dept. of biochemistry, Rutgers Univ., Peter Nissley Metabolism Branch, NCI

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Biochemistry

SECTION
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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
9.25	6.25	3.00

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of the project is to study the chemical and physical properties of proteoglycans and their biosynthesis. Topics of present interest include: 1) chemistry of proteoglycans isolated from the Swarm rat chondrosarcoma, 2) mechanisms of proteoglycan aggregation and interaction with hyaluronic acid, 3) biosynthesis of the protein core of proteoglycans, the link proteins, and hyaluronic acid by chondrocyte cultures, 4) relationship between proteoglycan structure and extracellular matrix organization using chick limb bud chondrocyte cultures, 5) characteristics and biosynthesis of proteoglycans in corneal stroma.

PHS-6040
(Rev. 10-76)

1. Project Description

Background

Cartilage proteoglycans are large macromolecules (MW 1-4 million) in which large, but variable, numbers of sulfated polysaccharide chains, chondroitin sulfate (CS) and keratan sulfate (KS), are covalently attached to a core protein. Such a molecular architecture yields macromolecules which occupy large hydrodynamic volumes in solution and which exhibit reversible compressibility, characteristics that help provide cartilages with resiliency and resistance to compressive forces. The core protein of cartilage proteoglycans consists of three distinct regions. One end, referred to as the HA-binding region, has a portion of protein (MW about 70-90 thousand) which is devoid of glycosaminoglycans and which interacts in a highly specific way with both hyaluronic acid (HA) and a protein (MW of 45,000) referred to as the "link" protein. These interactions are critical for the organization of proteoglycans into aggregate complexes, the predominant form of the proteoglycans in the tissue matrix. Adjacent to the HA-binding region is a portion of the core protein, referred to as the KS-enriched region (MW about 25-40 thousand), which contains an average of about 65% of the KS chains but less than 10% of the CS chains present in the intact molecules. Distal to the HA-binding region is a portion of the core protein, referred to as the CS-enriched region. This latter region has a variable molecular weight (from a few thousand to 200,000) and contains more than 90% of the CS chains but less than 35% of the KS chains present in the intact molecules. The variable length of this region appears to be proportional to the number of CS chains present on any individual proteoglycan molecule. The average proteoglycan molecule contains about 100 CS chains (average MW about 20,000 per chain) and 50 KS chains (assuming an average of about 5000 per chain).

This program collaborates with: 1) Dr. Dennis Torchia of this laboratory in studies relating to ^{13}C-NMR of ^{13}C-serine and ^{13}C-glycine labeled proteoglycans prepared from cultures of chick limb bud chondrocytes (Project # Z01 DE 00157-05), 2) Dr. Robert Gelman of this laboratory in studies relating to the interaction of proteoglycans with collagen during fibrillogenesis (Project # Z01 DE 00215-03), 3) Dr. Gretchen Hascall, Laboratory of Biological Structure, in studies on the morphology of the Swarm rat chondrosarcoma (Project # Z01 DE 00163-03), 4) Dr. Arnold Caplan in studies relating to the characteristics of proteoglycans isolated from the chick limb bud chondrocyte cultures (supported in part by a contract, NIDR # N01 DE-37042.) 5) Dr. John Hassell, Eye Institute, in studies on corneal proteoglycans. 6) Dr. Bo Nilsson, Visiting Fellow, NCI, in structural studies of oligosaccharides on cartilage proteoglycans. 7) Dr. Robin Poole in studies on the immunology of proteoglycans. 8) Dr. Peter Fietzek in studies on the protein chemistry of link protein and proteoglycan core protein. 9) Dr. Peter Nissley, Metabolism Branch, NCI, in studies on the effects of a somatomedin-like peptide and insulin on proteoglycan synthesis. The projects listed in last year's report as "3a Effects of β-xylosides on proteoglycans" and "4) Characteristics of proteoglycans isolated from porcine follicular fluid and synthesized by cultures of rat granulosa cells" have been completed.

The following sections describe our ongoing projects.

1. Characteristics of proteoglycans isolated from the Swarm rat chondrosarcoma.

Large quantities of proteoglycan aggregates can be purified from this transplantable rat chondrosarcoma without prior dissociation. The susceptibility of proteoglycan aggregate to digestion by the protease, clostripain, was studied. Several intermediate fragments from about 110,000 to 80,000 MW were observed for the core protein of proteoglycans during digestion. By limited digestion, a polypeptide of 65,000 MW, which contains an intact, functionally active HA-binding site, was obtained. The link protein, which remains functionally active, is reduced in size from about 44,000 MW to 40,000 MW by the digestion. Procedures were developed to separate and purify both the HA-binding region fragment of 65,000 and the link protein fragment of 40,000. Monospecific antisera have been raised against both of these components in rabbits. These antisera are currently being used for further structural work and for biosynthesis studies in chondrocyte cultures. Cyanogen bromide hydrolysis and specific enzyme digestion procedures are being developed for both the link protein and the HA-binding region polypeptide for more detailed studies of their protein structure.

2. Characteristics of proteoglycans synthesized in culture by chondrocytes from the Swarm rat chondrosarcoma.

a. Synthesis and function of link protein. Chondrocytes isolated from the tumor and grown in vitro synthesize proteoglycans characteristic of cartilage. ^{3}H-Serine was used to study the biosynthesis, secretion and the mechanism of aggregation of these proteoglycans. Proteoglycan aggregates isolated directly from the medium without prior dissociation contained about 8% of the label in link protein with the remainder in the core protein of proteoglycan. This proportion of label in link protein is consistent with a stoichiometry of one link protein for each monomer in aggregate structures. When unlabeled carrier monomer was added, additional labeled link molecules were recovered in aggregate fractions. Under the culture conditions, almost twice as many link protein molecules as monomers (mole/mole) were synthesized by the cells. While the addition to the medium of exogenous hyaluronic acid oligomers (long enough to bind both a link protein and a monomer) effectively prevented aggregation on macromolecular hyaluronic acid, the addition of a large excess of unlabeled monomer to medium was relatively ineffective in displacing labeled link molecules from labeled monomers. These results suggest that link molecules may associate with monomer molecules before binding to hyaluronic acid in the aggregation process.

b. Synthesis of hyaluronic acid. Procedures were developed, utilizing chondroitinase ABC digestion and separation of disaccharides on thin layer cellulose plates, to determine the kinetics of synthesis of hyaluronic acid relative to chondroitin sulfate in the cultures using ^{3}H-glucosamine as a precursor. The ratio of hyaluronic acid to chondroitin sulfate synthesized during a 24 hour period is about 1:12. The distribution of newly synthesized HA between cells, matrix and medium has been studied. Proportionally, in these chondrocyte cultures, more labeled HA than proteoglycan remains cell-associated suggesting the possibility that hyaluronic acid may serve additional functions near the cell in addition to forming the central filament for proteoglycan aggregates. Experiments are in progress to determine if hyaluronic acid is synthesized bound to a core protein using labeled amino acids as precursors and purifying the HA fraction.

c. Effect of cycloheximide on proteoglycan synthesis. Cycloheximide was used to block protein synthesis in chondrocytes. At times after the block, ^{35}S-sulfate was added to determine the kinetics of proteoglycan synthesis. Proteoglycan synthesis continues, but at an exponentially decreasing rate with a $t_{1/2}$ of about 90 minutes. The molecular characteristics of proteoglycans synthesized immediately after the cycloheximide block and at times up to 6 hours later are very similar except that the chondroitin sulfate chains gradually increase in size. The results suggest that there is a large precursor pool of core protein in the cells and that the cellular mechanisms for glycosaminoglycan biosynthesis and secretion of proteoglycans continue in the absence of new protein synthesis. This was further verified by pulsing cultures for 15 minutes with either ^{3}H-serine or ^{35}S-methionine, then introducing a cycloheximide block. Proteoglycans were then purified by dissociative gradients at subsequent times after the block. The appearance of both precursor amino acids in completed proteoglycans increased after the cycloheximide treatment with the same kinetics as the decrease in ^{35}S-sulfate incorporation into proteoglycans observed in the first experiment. Since ^{35}S-methionine is localized primarily in the HA-binding region and ^{3}H-serine primarily in the polysaccharide attachment region, these results are consistent with a single polypeptide core protein for proteoglycans. Attempts are underway to solubilize the intracellular core protein precursor pool and to identify and isolate these precursors as well as newly synthesized link protein using monospecific antisera directed against either link protein or the HA-binding region polypeptide.

d. Effect of a somatomedin (multiplication stimulating activity) and insulin on proteoglycan synthesis. Chondrocytes maintained in serum free Dulbecco's medium with high glucose for 4 days incorporate ^{35}S-sulfate into proteoglycans typical for cartilage but at a rate only about 25% that of cells grown in the same medium supplemented with fetal calf serum. Serum free Dulbecco's medium containing

0.5 μg/ml or higher concentrations of a peptide hormone with somatomedin activity increases the ^{35}S-sulfate incorporation into proteoglycans about 50% of the difference between medium alone and medium with fetal calf serum. Insulin is much more effective in stimulating proteoglycan synthesis with as little as 5 ng/ml giving a stimulation up to 80% of the difference. Experiments are underway to determine if the stimulatory effects of these polypeptide hormones are selective for proteoglycan synthesis and if the structures of the proteoglycans are altered.

3. Characteristics of keratan sulfate and oligosaccharides in proteoglycans (Section 3b of last year's report). Monomer proteoglycans from chick limb bud chondrocytes were treated with alkaline-borohydride to release glycosaminoglycans and oligosaccharides from the core protein. Combinations of molecular sieve chromatography, chondroitinase digestion and ion exchange chromatography were used to purify keratan sulfate, a mannose-rich oligosaccharide fraction and a group of small oligosaccharides. The keratan sulfate fraction contained proportions of sialic acid:glucosamine:galactose:galactosamine of 2:21:20:1. The galactosamine was recovered primarily as galactosaminitol indicating that the chains were attached to the core protein by O-glycoside bonds between galactosamine and the hydroxyl groups of serine and/or threonine residues. Less than 0.3 residues of mannose were present per galactosamine indicating that keratan sulfate probably does not contain mannose. The mannose residues in proteoglycans, previously reported by other investigators to be present in keratan sulfate, were shown to be located in a separate class of oligosaccharides with a composition of sialic acid:glucosamine:galactose:fucose:mannose of 0.5:1.3:0.8:0.1:1.0. No reduced sugar moieties were present after the alkaline-borohydride treatment suggesting that these oligosaccharides are probably linked to the protein through N-glycoside bonds between glucosamine and asparagine which are typical of glycoproteins. The small oligosaccharides contained galactosaminitol, and thus were linked to the core protein by the same type of linkage as the keratan sulfate chains. The proteoglycan from the rat chondrosarcoma, which does not contain keratan sulfate, was found to contain these small oligosaccharides as well as the mannose-rich oligosaccharides. The mannose-rich oligosaccharides were found to be located primarily, if not solely, in the 65,000 MW HA-binding region polypeptide. Molecular sieve chromatography on Biogel P-10 separated the small oligosaccharide fraction into three distinct oligosaccharides. The smallest was a trisaccharide with the structure

sialyl α(2 --→ 6) galactosyl (1 --→ 3) galactosaminitol.

The next larger was a tetrasaccharide

sialyl α(2 --→ 6) galactosyl (1 --→ 3) galactosaminitol
↑ α(2 --→ 6)
sialyl

2. Publications

Hascall, V. C. and Heinegård, D. K.: Structure of Cartilage Proteoglycans. Glycoconjugate Research: Proceedings of the 4th International Symposium on Glycoconjugates (J. D. Gregory and R. W. Jeanloz, editors) Academic Press (1979) 341-374.

Faltz, L. L., Reddi, A. H., Hascall, G. K., Martin, D., Pita, J. C., and Hascall, V. C.: Characteristics of proteoglycans extracted from the Swarm rat chondrosarcoma with associative solvents. J. Biol. Chem. 254:1375-1380, 1979.

Faltz, L. L., Caputo, C. B., Kimura, J. H., Schrode, J., and Hascall, V. C.: Structure of the complex between hyaluronic acid, the hyaluronic acid-binding region, and the link protein of proteoglycan aggregates from the Swarm rat chondrosarcoma. J. Biol. Chem. 254: 1381-1387, 1979.

Oegema, T. R., Hascall, V. C., and Eisenstein, R.: Characterization of bovine aorta proteoglycan extracted with guanidine hydrochloride in the presence of protease inhibitors. J. Biol. Chem. 254:1312-1318, 1979.

Yanagishita, M., Rodbard, D., and Hascall, V. C.: Isolation and characterization of proteoglycans from porcine ovarian follicular fluid. J. Biol. Chem. 254:911-920, 1979.

Heinegård, D. K. and Hascall, V. C.: The effects of dansylation and acetylation on the interaction between hyaluronic acid and the hyaluronic acid-binding region of cartilage proteoglycans. J. Biol. Chem. 254:921-926, 1979.

Heinegård, D. K. and Hascall, V. C.: Characteristics of the non-aggregating proteoglycans isolated from bovine nasal cartilage. J. Biol. Chem. 254:927-934, 1979.

Kimura, J. H., Hardingham, T. E., Hascall, V. C. and Solursh, M.: Biosynthesis of proteoglycans and their assembly into aggregates in cultures of chondrocytes from the Swarm rat chondrosarcoma. J. Biol. Chem. 254:2600-2609, 1979.

Caplan, A. I. and Hascall, V. C.: Structure and developmental changes in proteoglycans. (1979) Dilatation of the Uterine Cervix (F. Naftolin and P.G. Stubblefield, editors) pp. 79-98. Raven Press, New York.

Hascall, V. C.: Enamel protein biochemistry - Avenues for future success. J. Dent. Res. Special Issue B, Vol. 58, 825-828, 1979.

The largest had the same composition as the tetrasaccharide with an additional glucosamine. The glucosamine appears to be located on the 6-position of the galactosaminitol and it has a substituent on the 4-position. Since these oligosaccharides are located on the core protein where keratan sulfate is normally located and they have homology with the structure of the attachment region for keratan sulfate to the core protein, they quite likely are derivatives of the keratan sulfate attachment oligosaccharide structure which, for some reason, have not undergone chain elongation. The detailed structures of the largest oligossacharide and of the mannose-rich oligosaccharides are under investigation. The biosynthesis of these oligosaccharides by the chondrosarcoma chondrocytes is also being studied.

4. Characterization and biosynthesis of proteoglycans of corneal stroma. Radioisotopic precursors, ^{35}S-sulfate, ^{3}H-glucosamine and ^{3}H-mannose were used to study the synthesis of proteoglycans by Rhesus monkey corneal stroma explants in organ culture. Two proteoglycans were identified by molecular sieve chromatography and density gradient centrifugation. A chondroitin-dermatan sulfate proteoglycan with a molecular weight of about 150,000 was shown to contain one glycosaminoglycan chain (MW of $\sim$ 55,000) and a few oligosaccharides of small molecular weight per molecule. Unlike cartilage proteoglycans, about 60-70% of this proteoglycan consisted of protein. The second proteoglycan was smaller (about 70,000 MW) and contained keratan sulfate chains (MW of $\sim$ 7,000; 1-2 chains per molecule) as well as smaller oligosaccharides. Proteoglycans isolated directly from Rhesus corneas were shown to have the same structures. The explant cultures, then, synthesize proteoglycans with the same properties as those present in the tissue, and they can be used to study the synthesis and structure of corneal proteoglycans under a variety of experimental conditions.

Significance

Proteoglycans are major structural components of connective tissue. This is most obvious in cartilage where they in part determine physical properties and form, and are critical for normal skeletal function and development. Proteoglycans are also found in all other connective tissues, although sometimes only in small amounts. Their role in these tissues is in general not well understood, but is undoubtedly critical to function.

Proposed Course

The project will continue to investigate such parameters as the physical and chemical properties of proteoglycans, their interactions with other matrix molecules in the organization of an extracellular matrix, the mechanisms involved in their biosynthesis and catabolism, and the changes they undergo during tissue development and aging. A broad approach will be continued emphasizing not only basic physical and chemical studies but also the role of proteoglycans in biological systems.

Lohmander, L. S., Hascall, V. C. and Caplan, A. I.: Effects of 4-methylumbilliferyl-β-D-xylopyranoside on chondrogenesis and proteoglycan synthesis in chick limb bud mesenchymal cell cultures. J. Biol. Chem. in press.

Hassell, J. R., Newsome, D. A. and Hascall, V. C.: Characterization and biosynthesis of proteoglycans of corneal stroma from Rhesus monkey. J. Biol. Chem. in press.

Solursh, M., Hardingham, T. E., Hascall, V. C. and Kimura, J. H.: Separate effects of exogenous hyaluronic acid on proteoglycan synthesis and deposition in pericellular matrix by cultured chick embryo limb chondrocytes. Developmental Biology. in press.

Yanagishita, M. and Hascall, V. C.: Biosynthesis of proteoglycans by rat granulosa cells cultured *in vitro*. J. Biol. Chem. in press.

Caputo, C. B. and Raisz, L. G.: Degradation of Fetal Rat Cartilage Matrix in Organ Culture: Effect of *Streptomyces* Hyaluronidase. Connective Tissue Research. in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 DE-00157-05 LB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Biophysical Studies on the Structure of Connective Tissue

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI :	D. A. Torchia	Biophysicist	NIDR LB
OTHERS:	W. W. Fleming	NIH Postdoctoral Fellow	NIDR LB
	L. W. Jelinski	Staff Fellow	NIDR LB

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Dr. A. N. Schechter, NIAMDD

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SECTION
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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.45	2.40	2.05

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this project is to investigate the molecular structure of fibrous proteins and of proteoglycans and to study phosphorus chemistry in connective tissues. The structural information obtained will be correlated with function. Areas of present interest are 1) Structure of collagen and elastin. ^{13}C and ^{2}H magnetic resonance techniques are being used to study the structure and interactions in collagen and elastin fibers. 2) Proteoglycan structure. ^{13}C magnetic resonance is also being used to study the molecular mobility of the polysaccharide and protein chains in the chick limb bud proteoglycan monomer. 3) Phosphorus chemistry in connective tissue. Magic angle spinning ^{31}P magnetic resonance is being used to probe the structure of the phosphorus moieties in connective tissue. 4) ^{13}C magnetic resonance is being used to study the extent and mechanism of hemoglobin S gelation in cell-free preparations and in erythrocytes. For these studies, magnetic resonance spectrometers have been assembled which give ^{2}H, ^{13}C, and ^{31}P spectra of solids. High power decoupling, cross-polarization, magic angle spinning, and solid echo experiments are all performed.

PHS-6040
(Rev. 10-76)

Introduction

The goal of this work is to determine aspects of the molecular structure and interactions of macromolecules in connective tissue and to elucidate structure-function relationships.

Methods

The primary research tool employed is nuclear magnetic resonance (nmr). Until recently, high resolution nmr studies had been limited to flexible macromolecules since linewidths of rigid structures having high molecular weights were too broad to detect. However, structured molecules can now be studied in the solid state by decoupling the dipolar interactions between proton and ^{13}C or ^{31}P nuclei. Cross-polarization can be used to enhance ^{13}C or ^{31}P signals in rigid molecular lattices where long spin-lattice relaxation times make normal Fourier transform techniques impractical. In addition, cross-polarization can be combined with rapid spinning of the sample about the magic axis (an axis making an angle of 54.7° with the external magnetic field) to produce spectra having small linewidths, comparable to those obtained for samples in solution. In the case of ^{2}H nuclei, decoupling is not required and spectra can be measured directly using a pulsed echo technique.

We have built two pulsed nmr spectrometers which provide solid state spectra of ^{2}H, ^{13}C, and ^{31}P. High resolution cross-polarization spectra and normal Fourier transform spectra (of ^{13}C and ^{31}P) can be routinely obtained for samples ranging from inorganic crystals to whole tissues, and magic angle spinning spectra can be obtained for ^{31}P. Relaxation times in the laboratory and rotating frames, cross-polarization times, and chemical shift anisotropies can all be measured and provide information about molecular orientation and molecular motions covering the frequency range 10^{3} to 10^{10} Hz. Deuterium quadrupole coupling constants are obtained from ^{2}H spectra and are sensitive to molecular motions that occur on the 10^{4} - 10^{6} Hz timescale.

Unlike ^{31}P, the natural abundances of ^{2}H and ^{13}C are low (0.016 and 1.1%, respectively). Hence, it is advantageous to incorporate labeled amino acids into proteins under study. The presence of the label greatly simplifies interpretation of the spectra since the ^{2}H and ^{13}C resonances can readily be assigned to the labeled sites. We have used biosynthetic techniques to incorporate ^{2}H and ^{13}C labeled amino acids into collagen, elastin, and proteoglycans.

Progress

1. Collagen and Elastin Structure. Spectra of chick calvaria collagen fibrils containing a ^{13}C-labeled amino acid (Gly, Ala, Lys, Glu or Met) have provided strong evidence that rapid, anisotropic molecular motion occurs in the helix backbone and in the labeled sidechains. The

lineshape and quadrupole splitting observed for the ^{2}H-spectra of collagen fibrils labeled in the methyl group of Ala confirm the presence of backbone motion. These results are strong evidence that collagen fibrils are not stabilized by a unique set of interactions.

^{13}C-spectra of cross-linked elastin samples containing Val, Ala or Lys residues labeled at backbone carbonyl carbons have shown that the extensible regions of elastin have the kinetic freedom required of a viscoelastic rubber-like network. Elastin chain dynamics are sensitive functions of temperature and the degree of swelling. The opposing effects of these two variables make the chain dynamics of elastin virtually independent of temperature in the 20 - 40°C range. These results correlate with the macroscopic viscoelastic behavior of elastin which is also independent of temperature in this range.

2. Proteoglycan Structure. We have prepared samples of the chick limb bud proteoglycan monomer containing serine ^{13}C-labeled at C^{β} or glycine labeled at C^{α}. Linewidths and relaxation times measured for the labeled carbons indicate that the protein backbone and the point of attachment of the polysaccharide to the core protein undergo isotropic reorientation (as a consequence of segmental motions). This reorientation is 3-4 orders of magnitude faster than the motion calculated for the proteoglycan monomer as a whole. (N. B. This is a collaboration with Dr. V. C. Hascall, see project #Z01 DE-00134-05 LB.)

3. Phosphorous Chemistry in Connective Tissue using ^{31}P Magic Angle Spinning. We have just completed construction of a probe for spinning samples at the magic angle and have obtained preliminary high resolution spectra of hydroxyapatite and other solid samples containing ^{31}P.

4. ^{13}C Nmr of Hemoglobin S Gelation. We have shown that ^{13}C nmr provides a reliable means to quantitate the amount of polymer in a cell-free deoxygenated hemoglobin S gel. Preliminary studies, carried out on intact erythyocytes indicate that the nmr technique can be used to follow polymerization in the intact cell. (N. B. This is a collaboration with Dr. A. N. Schechter, NIAMDD.)

Significance

Interactions involving specifically labeled sites have been investigated using the new experimental technique of high resolution nmr in solids in conjunction with ^{13}C, ^{2}H, and ^{31}P-labeled tissues. New information about the molecular dynamics and interactions at specific sites in intact connective tissue has been obtained. This information has provided a basis for understanding the nature of the molecular interactions that determine the structure and function of the macromolecules investigated. In addition, ^{13}C nmr is a promising method for investigating potential inhibitors of gelation within intact red cells.

Future Plans

The general strategy of using solid state nmr to study labeled macromolecules will be followed. However, emphasis will shift from using ^{13}C labels to ^{2}H labels to probe protein structure and interactions. Hemoglobin S studies will concentrate on investigating the nature of hemoglobin gelation within the red cell. Magic angle spinning will be used to characterize phosphorous chemistry in connective tissues.

Publications

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J. W. H. Sutherland, W. Egan, A. N. Schechter and D. A. Torchia: Carbon-13-proton nuclear magnetic double-resonance study of deoxyhemoglobin S gelation. Biochemistry. 18:1797, 1979.

L. W. Jelinski and D. A. Torchia: ^{13}C/^{1}H High power double magnetic resonance investigation of collagen backbone motion in fibrils and in solution. J. Mol. Biol. 132, 1979. in press

C. T. Noguchi, D. A. Torchia and A. N. Schechter. ^{13}C Nuclear magnetic resonance quantitation of polymer in deoxyhemoglobin S gels. Proc. Nat. Acad. Sci, 1979. in press

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF **INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 DE-00215-03 LB

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Connective Tissue: Formation and Structure

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI :	R. A. Gelman	Staff Fellow	NIDR LB
OTHER :	K. A. Piez	Chief	NIDR LB

COOPERATING UNITS (if any)
Dr. Michael Beer, Johns Hopkins Univ.; Dr. Joseph Wall, Brookhaven National Laboratory; Dr. Ralph Nossal, DCRT

LAB/BRANCH
Laboratory of Biochemistry

SECTION
Protein Chemistry

INSTITUTE AND LOCATION
NIDR, NIH, Bethesda, Maryland 20205

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

It is the long range purpose of this project to study interactions and relationships between connective tissue macromolecules as a way to understand connective tissue formation and structure. The topics of present interest are: 1) The mechanism of collagen fibril formation in vitro. 2) High resolution electron microscopy of fibrils and early aggregates in collagen assembly. 3) Quasielastic light scattering. 4) The effect of noncollagenous molecules on collagen assembly in vitro. We have developed a reproducible in vitro assembly system and shown that collagen fibril formation is a multistep process. After an early initiation step that is not understood, linear assembly of thin filaments dominates, followed by lateral association of the filaments to form native collagen fibrils.

PHS-6040
(Rev. 10-76)

Project Description

Objectives

1. The mechanism of collagen fibril formation in vitro. The development of a well-characterized, reproducible system for the study of collagen fibril formation in vitro and of models of fibril structure has made it possible to investigate the mechanism of collagen assembly in greater detail than previously possible. The dependence of assembly on collagen concentration and temperature has been established allowing the manipulation of assembly and the isolation of separate stages for individual study.

2. High resolution electron microscopy of collagen fibrils and early aggregates in assembly.

The properties of connective tissues are determined in part by the packing of collagen molecules into functional units, the native fibril. The precise relationship between monomers in a highly ordered fibril-for example, a tendon--is not known. The longitudinal relationship is known but information regarding lateral placement, supercoiling and possible subfibrillar units has been limited by the methods available. Electron microscopy has the potential of providing information down to at least 2 nm if certain instrument modifications are utilized and preparation of the specimen both conserves native structure and optimizes electron density differences. The major purpose of this aspect of the project is to obtain improved electron microscopic information through improved techniques and the use of in vitro aggregates of collagen.

3. Quasielastic light scattering.

Quasielastic light scattering affords a technique for the study of the size and shape of macromolecules in solution. This technique should prove to be well suited for studies of the early stages of collagen assembly where small intermediates are believed to form. Development of instrumentation has been stimulated by this problem. The application of the technique in investigations into the structure of other biological macromolecules is also of interest.

4. The role of noncollagenous molecules in collagen assembly and fibril structure.

In vivo, collagen assembles in the presence of a variety of macromolecules and low molecular weight substances. Although none of these (except perhaps phosphate) appears to be necessary for in vitro assembly, it is likely that they may be involved in vivo, perhaps to regulate the process. Of particular interest are proteoglycan, lysyl oxidase and fibronectin. We initially propose to study the effect of proteoglycan on in vitro assembly.

Methods Employed

Type I collagen is prepared from rat tail tendon, and purified and characterized by standard biochemical and biophysical methods. Fibril structure and kinetics of formation are being studied by electron microscopy, turbidimetry, and quasielastic light scattering. Increased resolution of electron micrographs is being sought by systematically investigating various preparative methods for specimens and by utilizing techniques to minimize beam damage. In addition to conventional transmission electron microscopy (CTEM), we are utilizing scanning transmission electron microscopy (STEM) at Johns Hopkins to visualize heavy atom-labeled collagen and at Brookhaven for mass measurements of collagen aggregates. A quasielastic light scattering instrument has been built and is fully operational. In some studies (only where noted) Type II collagen from rat chondrosarcoma has also been used.

Progress

1. The mechanism of collagen fibril formation.

A set of optimal conditions for the self-assembly process has been selected and used in an investigation of the mechanism of collagen assembly. We have found that collagen assembly occurs via a mechanism of at least three steps. Step 1, initiation, involves a temperature-dependent change which leads to an unidentified intermediate that in the second step spontaneously grows. Step 2 is linear growth of filaments by a temperature-independent process. Step 3 is lateral association of filaments by a temperature-dependent process. The concentration dependence of the rates of steps 2 and 3, and the lack of a measurable critical concentration suggest assembly by accretion. We have shown that step 1 is complete in 6 min at 26° and is independent of collagen concentration. This result suggests a rate limiting conformational change but does not rule out some form of early aggregation. Collagen treated with pepsin, which removes the nonhelical ends, makes distorted fibrils by a similar mechanism to normal collagen. However, assembly is very much slower and step 1 is markedly altered. The nonhelical ends therefore are critically involved in assembly.

2. High resolution electron microscopy of collagen aggregates formed early in assembly.

Native collagen fibrils and reconstituted collagen fibrils have been examined by electron microscopy using standard methods. Observation of collagen in various stages of assembly and under various conditions has shown that intermediate aggregates are formed early in assembly (step 1) and that the formation of long thin filaments (step 2) precedes banded fibril formation (step 3). These intermediate aggregates and their filaments are difficult to observe and must be distinguished from artifacts. Preliminary investigations suggest that three kinds of high resolutions studies are potentially useful in this problem. (a) CTEM of negatively stained preparations using instrumental procedures for minimal radiation damage. (b) STEM of preparations labeled with single heavy atoms at specific sites. The arrangement of collagen molecules in an aggregate can be deduced from the pattern of heavy atoms. (c) STEM of unstained preparations to determine mass. For example, a knowledge of mass as a function of length of the thin filaments formed in step 2 will be important in determining structure.

3. Quasielastic light scattering.

Computer programs designed to analyze the data obtained from the light scattering instrument have been written; analysis involves use of a non-linear regression routine which utilizes an on-line computer. The direct result obtained is the diffusion coefficient of the species (molecule or aggregate) in the solution being studied. From hydrodynamic theory it is then possible to calculate the size and shape of the molecule or aggregate.

As a control, we initially studied collagen in acidic solution and confirmed the published value for the diffusion coefficient and its anomalous concentration dependence. We found that in neutral solution at 4°C the concentration dependence was normal and that our collagen preparations are monomeric under these conditions. When fibril formation was initiated by raising the temperature, the diffusion coefficient dropped rapidly over 5-10 min and then reached a constant value. This result is consistent with our conclusions regarding steps 1 and 2 in assembly, described above, and places constraints on the structures involved.

In collaborative studies with Dr. R. Nossal, the light scattering instrument was used to study the mechanical properties of gels.

4. The effect of proteoglycan on collagen assembly _in vitro_.

We have confirmed previous reports that the rate of type I collagen assembly is retarded in the presence of a cartilage-type proteoglycan from a rat chondrosarcoma. Proteoglycan also binds tightly to the collagen but does not appear to alter the structure of the fibril. On the other hand, proteoglycan slightly accelerates the assembly of type II collagen and does not bind. Since type I and II collagens are basically similar molecules and form similar fibrils, this result argues for a highly specific interaction between collagen and proteoglycan. It seems unlikely that the effect of proteoglycan depends on some non-specific property such as its polyanionic character.

Significance

The basic properties of connective tissues can be described in terms of the macromolecules of which they are composed. Of these, collagen is the major structural protein. Collagen is present as native fibrils which vary in diameter and in their higher level organization as a function of species, tissue and developmental stage. Information regarding the mechanism of assembly and interactions with other macromolecules provides a basis for continued research on development in tissues and on the relationship of structure to disease.

Proposed Course

1. Continuing emphasis will be placed on high resolution electron microscopy to resolve structural questions related to fibril formation. Of particular interest is the structure of early intermediates and of the thin filaments that we observe. We expect that computer processing methods developed as part of another study will be useful in analyzing electron micrographs.

2. Our studies of the mechanism of *in vitro* assembly of collagen will be continued using collagen types other than type I. We will also investigate the role of other macromolecules in the process.

3. Because of personnel and space constraints, the quasielastic light scattering instrument will be transferred to another Institute at NIH where it will be used on other projects. It will also be available to us for use on this project.

Publications

Williams, B. R., Gelman, R. A., Poppke, D. C. and Piez, K. A.: Collagen fibril formation. Optimal *in vitro* conditions and preliminary kinetic results. *J. Biol. Chem.* 253:6578-6585, 1978.

Gelman, R. A., Williams, B. R. and Piez, K. A.: Collagen fibril formation. Evidence for a multistep process. *J. Biol. Chem.* 254:180-186. 1979.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 DE-00001-28 LB

PERIOD COVERED
October 1, 1978 - September 30, 1979

TITLE OF PROJECT (80 characters or less)
Transglutaminases: Specificities, Physiological Functions and Catabolism of Products

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI. :	J. E. Folk	Chief, Enzyme Chemistry Section	NIDR LB
	M.L. Fink	Staff Fellow	NIDR LB
	J. Schrode	Staff Fellow	NIDR LB
Others :	J.J. Gorman	Visiting Fellow	NIDR LB
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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Specificity studies on transglutaminases using synthetic model substrates are underway. The relationship to cellular control processes and other biological processes of polyamine-protein conjugates produced by these enzymes in cells and body fluids is under investigation. Knowledge of the catabolism of these conjugates has been obtained.

PHS-6040
(Rev. 10-76)

1. Project Description

Objectives

Studies carried out over the past several years have been directed toward characterization of enzymes termed transglutaminases that are responsible for the formation of γ-glutamyl amide bonds in proteins and polypeptides. These enzymes catalyze transfer reactions at the carboxamide group of peptide-bound L-glutamine with a high degree of stereospecificity. In the presence of acceptor amines, the transfer reaction results in the formation of substituted amides ($-COHN_2 + RNH_2 \rightarrow -COHNR + NH_3$).

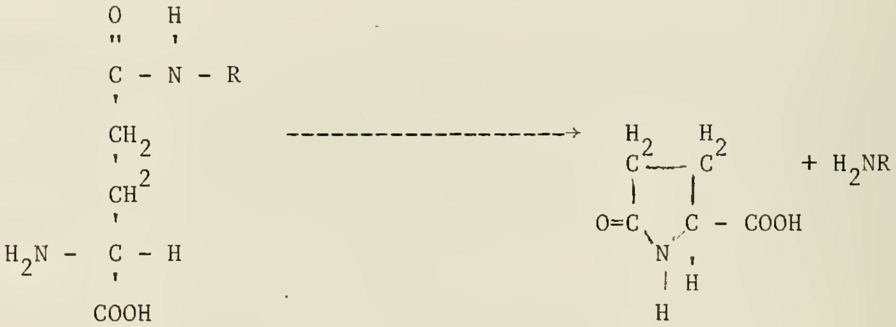
One of the well-characterized covalent crosslinks between and within protein molecules is the ε(γ-glutamyl)lysine bond. There has been increasing evidence for the presence of this crosslink in fibrin clots, cell membrane, glycerinated myofibrils of muscle, proteins of seminal plasma, native wool keratin, and the citrulline-containing protein fraction of hair. We have recently observed that the transglutaminases can catalyze formation of a hitherto undescribed crosslink. This crosslink between peptide chains is formed by means of a transfer reaction between the carboxamide group of a glutamine residue in each chain and both primary amino groups of a diamine or polyamine. The wide occurence and obvious importance of γ-glutamyl amide bonds have led us to focus attention on the enzymes responsible for their formation, as well as on the occurrence and functions of these bonds in various proteins.

This project is in part collaborative with S. I. Chung project number Z01 DE-00049-08 LB.

Major Findings

The specificity of activated human blood coagulation factor XIII is being systematically investigated. Since, it was known that mixed casein is an excellent substrate for this enzyme, purified αls -, β- and κ-caseins isolated from the milk of cows homozygous for single variants of these caseins were examined as substrates. β-casein, A variant was found to be by far the best substrate.

β-casein was labeled with a fluorescent amine substrate by use of factor XIIIa and the labeled casein, containing a single derivatized glutamine, was subjected to chemical and enzymic degradation. Peptide mapping and sequence studies identified the glutamine residues as Gln-167. Solid phase synthesis of a 15 member peptide containing the sequence of β-casein surrounding this glutamine residues was carried out. This peptide was found to be an excellent substrate for partially purified from rabbit kidney. The enzyme is a cyclohydrolase catalyzing the breakdown in the following way:



Significance

Understanding of the molecular characteristics of the transglutaminases is vital to determination of the function of these enzymes in normal and diseased tissues, pharmacological control of activity, and hormonal regulation.

The minimal substrate structural requirements for transglutaminases have been defined over the past several years through studies such as those described. We now have a better understanding of the mechanism of enzyme-substrate interactions in transglutaminase-catalyzed reactions. The techniques used for these studies, as well as ones used in determining specificity toward glutamine and lysine residues in macromolecular substrates, most certainly will show similarities, as well as differences, in the members of this important group of enzymes.

Both polyamines and transglutaminases are widely distributed and both may play important roles in control of growth and other biological processes. The finding that polyamines are natural substrates for these enzymes is an important first step in determining the role each plays in these vital processes. That there may be differences in the manner in which the polyamines serve as substrates in cells and in body fluids may contribute to our understanding of their possible function in control of cellular and extracellular processes.

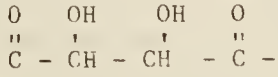
Until now little has been known about the catabolism of products of transglutaminase action. The characterization of and determination of the specificity of the enzyme that breaks down γ-glutamyl lysine and polyamine derivatives will certainly be of value in understanding this vital process.

factor XIIIa. Approximate 20 peptides containing variation in this sequence were prepared and tested as substrates. The findings supply evidence that several aliphatic hydrophobic amino acid residues on each side of the substrate glutamine are essential for optimum substrate properties and that a lysine residue close to the glutamine is also essential. These results form the basis for design of excellent low molecular weight substrates for factor XIIa.

A transglutaminase-mediated procedure for photochemical labeling of peptides and for production of cleavable crosslinks between protein molecules has been devised. Use of bifunctional amine substrates of the general structures:

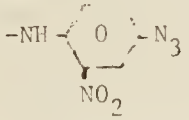
$$H_2N - (CH_2)_2 - R_1 - (CH_2)_2 - R_2$$

where R_1 = -S-S-,



$\overset{O}{\overset{\|}{C}} - \overset{OH}{\overset{|}{CH}} - \overset{OH}{\overset{|}{CH}} - \overset{O}{\overset{\|}{C}}$ - or -

and $R_2 = NH_2$ or



have been employed to prepare photolabeled peptide hormones for receptor studies and have been used for reversible crosslinking of model proteins. These reagents should extend the usefulness of transglutaminase as tools in protein and peptide chemistry.

A fragment of factor XIII $(\underline{a}_2\ \underline{b}_2)$ noncatalytic b subunits has been isolated from cyanogen bromide digests of this subunit. This fragment retains the structural features essential for binding to a chains, but has a molecular weight only about one-half that of native b chain, contains only a portion of the original carbohydrate, and is converted to several peptides upon reduction. A recombinant formed with a subunits is more labile to chemical modification than is native factor XIII.

Evidence has been accumulated that putrescine and the polyamines, spermidine and spermine, serve as physiological substrates for transglutaminases in both cells and body fluids. Crosslinks between proteins through polyamines occur in the extracellular system tested; i.e., during clotting of rat seminal fluid. In human peripheral lymphocytes, although no evidence of crosslinking was obtained, protein conjugates of both putrescine and spermidine were found after treatment of cells with nitrogens. In both systems the polyamines were found to be complexed through covalent γ-glutamyl linkage, strong evidence for their transglutaminase-catalyzed incorporation.

An enzyme that catalyzes the breakdown of ε-(γ-glutamyl)lysine, N-(γ-glutamyl) polyamines and N,N-bis-(γ-glutamyl)polyamines has been

Proposed Course

Specificity studies will be continued with special emphasis placed on the transglutaminases involved in hemostasis and wound healing. Principal objectives will be to determine the importance of amino acid side chains in close vicinity to substrate glutamine residues and, thus, to design effective small substrates for the enzymes.

Now that strong evidence has been obtained that the polyamines function as transglutaminase substrates, attempts will be made to determine how the products of these reactions participate in cellular and extracellular processes. First steps will involve characterization of the protein conjugates, identification of their cellular or extracellular location, and estimation of their metabolic rates and characteristics.

Knowledge of the specificity and distribution of the enzyme that breaks down γ-glutamyl derivatives should reveal much information about the basic catabolism of the products of transglutaminase action. For example, can crosslinks be hydrolyzed in intact proteins or must the proteins be largely degrated before the crosslinks can be cleaved? The answer to this question is of prime importance to a thorough understanding of the physiological role of these important enzymes.

2. Publications

Schrode, J., and Folk, J.E.: Transglutaminase-catalyzed crosslinking through Diamines and Polyamines. J. Biol. Chem. 253,1978.

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Gorman, J.J., and Folk, J.E.: Structural features of glutamine substrates for human plasma factor XIIIa. J. Biol. Chem. in press.

Gorman, J.J., and Folk, J.E.: Transglutaminase amine substrates for photochemical labeling and cleavable crosslinking of proteins. J. Biol. Chem. in press.

ANNUAL REPORT
LABORATORY OF BIOLOGICAL STRUCTURE
NATIONAL INSTITUTE OF DENTAL RESEARCH

The research efforts of the Laboratory of Biological Structure during the past year have continued to focus on the elucidation of the structural, chemical and functional characteristics of the hard and soft tissues of the oral cavity. Although no major new initiatives have been undertaken, considerable progress has been made through the logical extension of our existing research projects. These results are summarized below by the general area of investigation, and are presented in detail in the narrative statements of the individual progress reports.

Apatite Structure and Formation

Studies of the chemistry, structure and formation of hydroxyapatite (HAP) and related calcium phosphate salts have concentrated in two main areas: quantitation of the hydroxide content of apatite; and the characterization of calcium phosphate precipitation from seeded solutions. Previous results suggested that the early crystalline phases formed during the *in vitro* precipitation of calcium phosphate were deficient in hydroxide. A titration method for direct analysis of the hydroxide content of the precipitates was developed. The earliest formed crystalline phases contained 0-20% of the theoretical amount of hydroxide for HAP, while the most mature apatites studied rarely contained greater than 60% of the theoretical hydroxide content. In conjunction with the titration method, procedures for quantitation of hydroxide content were established using infrared and laser Raman spectroscopy. Analysis by these methods agreed within -11% to +18% and ± 20%, respectively, of the chemically determined hydroxide content. These spectroscopic procedures are under systematic evaluation to establish conditions for maximum reliability. The significance of the reduced hydroxide content is not clear, but it indicates that hydroxide is not required for stability of HAP under simulated biological conditions.

Since most biological calcification occurs in the presence of preexisting apatite crystals, studies of calcium phosphate precipitation reactions focused on precipitations induced by seeding stable supersaturated solutions with apatite crystals. The course of the reaction depended upon the initial degree of supersaturation. With calcium and phosphate concentrations equal to or greater than 1.2 and 0.9 mM, respectively, an octacalcium phosphate-like intermediate occurred, as in spontaneous precipitation reactions. In contrast, with lower initial calcium and phosphate concentrations, an octacalcium phosphate phase did not occur. Previous studies of spontaneous precipitation reactions demonstrated that F^- and Mg^{2+} affected the lifetimes of the various intermediate phases. In seeded reactions, F^- retarded reaction development at high supersaturations, while it accelerated the initial reaction at low supersaturations. The effects of F^- on the crystallinity of apatites was also studied. Whereas in fluoridated bone the crystals are increased in width and thickness but not in length, in *in vitro* systems fluoride favored the formation of narrow needle-like crystals. These results emphasize the importance of biological factors, such as local calcium and phosphate concentrations and extracellular matrix macromolecules, in controlling the growth of apatite crystals *in vivo*.

Tooth Matrix Proteins and Mineralization

Studies of the matrix proteins of fetal bovine teeth have extended the observations reported last year on the chemistry and structure of these unusual molecules. The amelogenins, a series of 8-10 proline-rich proteins, are found only in fetal enamel matrix. With increasing fetal age or degree of mineralization a shift from higher to lower molecular weight species occurred. The amelogenins contained 1% sialic acid, 1% hexosamine and 10-15% total carbohydrate. The fetal enamelins are the probable precursors of the mature enamel matrix proteins. They were extracted in higher proportion (relative to amelogenins) from older fetal teeth than from younger teeth, and the data suggest that they are synthesized at all stages of enamel development. The enamelins were phosphorylated, and contained 4-6% sialic acid, 4-5% hexosamine and about 20% total carbohydrate. The biogenesis of these enamel matrix proteins is being studied in hamster molar tooth germs grown in culture for 2-4 days.

The dentin specific proteins have been found to consist of a major phosphoprotein of 96,000 Daltons, comprising one-half to two-thirds of the non-collagenous matrix proteins, 3 phosphorylated glycoproteins in the 30-80,000 Dalton range, and 2-3 phosphorylated glycoproteins in the 10-25,000 Dalton range. The major dentin phosphoprotein previously has been shown to inhibit apatite formation from synthetic calcium phosphate solutions. Pretreatment of the phosphoprotein with ionic calcium and/or collagen fibrils reduced the inhibitory effects on apatite formation. The phosphoprotein also inhibited the crystal growth processes in seeded precipitation systems. While pretreatment with calcium diminished the inhibition, no effect was seen after pretreatment with collagen. The dentin phosphoprotein also inhibited *in vitro* collagen fibrillogenesis; this inhibition was greatly enhanced by pretreatment with calcium. These results suggest an important regulatory role for the dentin phosphoprotein in dentinogenesis and dentin mineralization.

In a continuation of studies initiated last year microprobe methods are being used to characterize *in situ* the organic matrix and initial mineral deposits in enamel, dentin and bone. These methods allow correlation of molecular and elemental characteristic with the histological structure. In the enamel matrix, the majority of the protein appeared to be in an antiparallel β-pleated shett configuration. Additionally, an adenosine and/or guanosine nucleoside has been identified. In all three tissues, spectra obtained from the earliest inorganic deposits were suggestive of the magnesium-carbonate containing mineral, huntite [$Mg_3Ca(CO_3)_4$]. The earliest detectable phosphate was apatitic in nature, with no evidence obtained as yet of precursor phases. Studies to extend these observations are currently underway.

Endochondral Bone Differentiation

The use of the in vivo matrix-induced bone differentiation system has continued to yield significant data concerning the processes of cartilage and bone cell differentiation and matrix-cell interactions. A series of experiments established the local mitogenic effect of the implanted collagenous matrix on the host mesenchymal cells. Increases in ornithine decarboxylase, a marker enzyme for cell proliferation, and incorporation of ^{3}H-thymidine were observed on days 3 and 8-9 after implantation. Radioautography revealed that the cells labeled with ^{3}H-thymidine were located close to the particles of implanted matrix. In a related study, it was found that when the crosslinking of newly-synthesized collagen was inhibited by β-aminopropionitrile, chondrogenic differentiation was inhibited and the mineralization of cartilage and bone was greatly reduced. These results attest to the importance of the collagenous matrix for cell growth and differentiation, and are a first step in understanding the complex interrelationships between connective tissue cells and their surrounding matrix.

A variety of nutrients, hormones, metabolites, drugs and other substances interact directly or indirectly with skeletal tissues. For example, periodontal disease is known to be complicated by diabetes, but the precise role of insulin in the differentiation and metabolism of skeletal tissues is unknown. Since the matrix-induced system is ideal for studying the influence of these substances on the various stages of cartilage and bone formation, the effects of experimental diabetes and insulin on the development of endochondral bone were studied. The results indicated a marked inhibition of mesenchymal cell proliferation on day 3 after implantation in diabetic animals. The reduced cell proliferation resulted in a decreased and delayed formation of cartilage. The vascular invasion prior to chondrolysis and osteogenesis was also decreased and delayed. Finally, diabetes caused a reduction in alkaline phosphatase activity and ^{45}Ca incorporation during cartilage calcification and osteogenesis, which could be prevented by administration of exogenous insulin.

Structure and Function of Secretory Cells

Progress in the studies of secretory cells during the past year has occurred in two main areas: Golgi apparatus-GERL structure and relationships; and identification and function of components of the lysosomal system. Acid phosphatase and thiamine pyrophosphatase have previously been utilized as enzyme markers of GERL and the Golgi saccules, respectively. Under certain conditions, however, the specificity of thiamine pyrophosphatase for the Golgi saccules was altered; reaction product was present in GERL and forming secretory granules as well as the Golgi saccules. In other cells GERL is frequently considered to be specialized endoplasmic reticulum, but the present results, which are consistent with previous observations on exocrine cells, suggest that GERL may be more closely related to the Golgi saccules than to the endoplasmic reticulum. An important goal of these morphological studies is the elucidation of the mechanisms of secretory protein transport and packaging. The localization of neurophysin, the carrier protein for vasopressin and oxytocin, was determined in the neurosecretory neurons of the supraoptic nucleus, using immunocytochemical procedures. Neurophysin was present in the organelles associated with protein synthesis and transport, but it was not detectable in GERL, from which the neurosecretory granules form. Although these findings parallel those previously reported for lacrimal acinar cells, the inability to detect secretory proteins in GERL is curious. While many factors may be involved a likely explanation is the inability of the cytochemical reactants to penetrate the membrane of GERL.

The lysosomal system has many functions in secretory cells. In addition to the usual participation in the cellular economy through degradation of effete macromolecules and organelles, under certain conditions lysosomes sequester and degrade secretory granules, and participate in the retrieval of excess membrane from the cell surface following exocytosis. An additional function studied in exocrine acinar cells and the neurosecretory neurons is the uptake of intravenously administered exogenous materials. Despite the marked differences in the two cell types, some striking similarities existed in the uptake and sequestration processes. In the neurons horseradish peroxidase (HRP) was taken up in endocytic organelles, and transported retrogradely to the cell body where it accumulated in secondary lysosomes. Cisternae of agranular reticulum, in continuity with the lysosomes, appeared to function in anterograde transport of the HRP and acid hydrolases. In exocrine cells, in addition to endocytic organelles and secondary lysosomes, HRP was sequestered in elongated basally-located cisternae. These cisternae reacted positively for a lysosomal marker enzyme, trimetaphosphatase, although they were generally unreactive for the classical lysosomal marker, acid phosphatase. Thus, in both neurosecretory neurons and exocrine acinar cells, cisternal structures involved in the uptake, sequestration and transport of exogenous materials have been identified cytochemically as part of the lysosomal system.

The interest of the laboratory in the secretory process and the commitment of NIDR to basic biological research led to the organization of a conference on Basic Mechanisms of Cellular Secretion, which was held in Annapolis, Maryland, September 17-21, 1979. The program focused on seven major topics related to protein secretion, and featured formal presentations by about 40 invited speakers and three poster sessions of contributed papers. A total of about 200 scientists participated in the conference. The co-organizers of the conference were Drs. Arthur R. Hand and Constance Oliver, and secretarial service was provided by Mrs. Patricia Youmans.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF
HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DE 00028-12 LBS

PERIOD COVERED

October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Ultrastructure and Cytochemistry of Secretory Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Hand, Arthur R.	Chief, Laboratory of Bio. Str.	LBS NIDR
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	Floyd, Steven W.	Photographer	LBS NIDR

COOPERATING UNITS (if any) Dr. Richard D. Broadwell, LNNS, NINCDS and Dr. Robert M. Greene, Department of Anatomy, Jefferson Medical College, Philadelphia, Pa.

LAB/BRANCH

Laboratory of Biological Structure

SECTION

Experimental Morphology Section

INSTITUTE AND LOCATION

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
6.20	3.79	2.41

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Basic mechanisms of the secretory process are studied in cells of the rat pancreas, salivary and lacrimal glands. Techniques utilized include light and electron microscopy, cytochemistry, radioautography, and biochemical determination of enzyme and cyclic nucleotide levels. Major areas of investigation are: (1) the role of the Golgi apparatus and GERL in secretory granule formation; (2) the structure and function of GERL and cyclic nucleotide levels in secretory cells of the beige mouse, a homologue of the human Chediak-Higashi syndrome; (3) the role of lysosomes in the intracellular degradation of secretory material and cellular membranes; and (4) the effects of sialographic procedures on the structure of the rat submandibular gland.

PHS-6040
(Rev. 10-76)

1. Project Description

Objectives:

The basic objective of this project is to obtain further knowledge of the structure and function of secretory cells and their organelle systems. Utilizing electron microscopic, cytochemical, radioautographic and biochemical techniques, cell ultrastructure is correlated with enzyme localization, quantification of cellular constituents, and glycoprotein synthesis and transport. Our efforts have concentrated on: (1) the role of the Golgi apparatus and GERL in the transport and packaging of secretory material; (2) the function of lysosomes in the degradation of secretory material and cellular membranes; and (3) the effects of sialographic procedures on the structure of the rat submandibular gland.

Methods Employed:

Tissues for morphological examination are fixed by vascular perfusion and prepared by standard techniques. Cytochemical incubations are carried out on 50-75 μm slices of fixed tissue. Radioautographs of tissue labeled with tritiated glycoprotein precursors are prepared by the method of Kopriwa (1973). The procedure of Nadler (1971) is utilized for grain counting and statistical analysis of EM radioautographs. Biochemical determinations of protein, DNA, amylase, peroxidase, and other enzyme activities follow standard procedures. Cyclic nucleotide levels are determined by radio-immunoassay.

Major Findings:

Our previous studies of exocrine secretory cells demonstrated that GERL, an acid phosphatase containing smooth membrane system located in the inner Golgi region, is intimately involved in the formation of secretory granules. The membrane of GERL is continuous with that of the forming granules, and acid phosphatase reaction product is present in the forming granules. In contrast, secretory proteins, as judged by the localization of endogenous peroxidase, are present in the Golgi saccules and forming granules but usually are not present in GERL. These cytochemical results raise questions concerning the route of secretory protein transport through the various components of the Golgi region, the origin of GERL and its precise relationship to other organelles, and the function of acid hydrolases in secretory granules. The following studies are part of a continuing effort to obtain additional information on these and other questions related to the secretory process.

Cytochemical studies of the Golgi apparatus and GERL have been pursued using three different systems. The apparent role of GERL in secretory granule formation suggested that changes in the structure and cytochemistry of GERL might be expected when the rate of granule production was altered. Animals were injected with either cholinergic (pilocarpine) or β-adrenergic (isoproterenol) agonists to induce granule discharge and a subsequent resynthesis of new granules. Lacrimal and parotid glands from animals sacrificed at various times after injection are currently being examined to determine the nature and extent of changes in the Golgi apparatus and GERL. A preliminary observation made on the parotid acinar cells of young animals suggested that the localization of thiamine pyrophosphatase activity was significantly different than that found in adult animals. Thiamine pyrophosphatase and acid phosphatase localizations are being studied in animals of varying ages from 2 weeks to adult. In the young animals, TPPase reaction product is present in the trans Golgi saccules, as in the adult, but is also frequently present in structures morphologically identified as GERL and forming secretory granules. Acid phosphatase is also present in GERL and forming granules. These findings suggest that GERL is closely related to and may be derived from the Golgi saccules, rather than the endoplasmic reticulum. The third system utilized is the neurosecretory neuron of the supraoptic nucleus of the hypothalamus. These neurons produce the peptide hormones vasopressin and oxcytocin, which are synthesized in the cell body, packaged into granules by the Golgi apparatus and GERL, and transported down the axon to the neurohypophysis. The neurons can be stimulated experimentally by adding 2% NaCl to the animal's drinking water. The localization of neurophysin, the carrier protein for the peptide hormones, was determined by light and electron microscopy using immunocytochemical procedures. Light microscope sections were used to establish antibody specificity and optimal staining conditions. By electron microscopy reaction product indicating the presence of neurophysin was localized in the nuclear envelope, endoplasmic reticulum, Golgi saccules, secretory granules and lysosomes. No labeling of GERL was observed. These results are consistent with our findings for secretory proteins in other cells. The lack of reaction product in GERL may in part be due to the inability of the large antibody molecules to penetrate the membrane of GERL.

Glycoprotein localization and transport in secretory cells is also being studied by electron microscope radioautography of cells labeled in vivo with the glycoprotein precursor ^{3}H-fucose. It is hoped that these studies will provide information on the movement of labeled secretory material through the Golgi saccules and GERL, although the resolution of the radioautographic method along with other technical difficulties may prove to be the limiting factors. Initial results on parotid acinar cells demonstrate heavy labeling of the Golgi saccules up to 40 minutes after injection of ^{3}H-fucose; GERL also appears labeled, but to a lesser degree. Analysis of the grain counts, and determination of the relative concentration of label in the various organelles have not been completed. Similar studies will be carried out on the exorbital lacrimal acinar cells, and possibly the serous demilune cells of the sublingual gland.

Preliminary EM radioautographic results on fucose uptake by pancreatic islet cells confirmed that the insulin producing β-cells are much more heavily labeled than other islet cells. The localization of grains after fine-grain development procedures suggests that the fucose is probably incorporated into a membrane glycoprotein. Further characterization may prove this fucose-labeled component to be a useful marker for β-cell plasma membranes, as well as an experimental model to study membrane biogenesis and recycling in a secretory cell.

The morphology and function of the lysosomal system of secretory cells has also been studied. The neurosecretory neurons of the supraoptic nucleus have been utilized to investigate the relationship of the agranular reticulum to the lysosomal system. Horseradish peroxidase (HRP), administered intravenously, is initially sequestered in small vesicles and cup-shaped organelles of the axon terminals, and then transported retrogradely to the cell body. In the cell body these endocytic organelles fuse with secondary lysosomes. HRP reaction product has never been observed in Golgi saccules or GERL. The secondary lysosomes are directly connected to cisterns which are morphologically similar to the agranular reticulum. These cisterns appear to provide a route whereby HRP and lysosomal constituents may be transported anterogradely. Furthermore, on the basis of its hydrolase activity, at least a portion of the agranular reticulum must be considered as part of the lysosomal system. Another animal model used to study lysosomal function is the beige mouse, a homologue of the human Chediak-Higashi syndrome. The defect in these animals is manifested by greatly enlarged lysosomes in many cell types and abnormal release of blood cell granules. Lowered intracellular levels of cyclic GMP has been proposed as the underlying cause of this defect. The levels of both cyclic GMP and AMP were compared in 10 tissues from beige and C-57 black mice. No consistent differences were noted among the tissues. However, lowered levels of both cGMP and cAMP were detected in the exorbital lacrimal gland of the beige mice. Based on these results, a study has been initiated to correlate the time course of secretory granule discharge from the lacrimal acinar cells with cyclic nucleotide levels following cholinergic (pilocarpine) stimulation.

In a previous study we had followed the distribution and uptake of exogenous tracers introduced via retrograde infusion of the main excretory duct of the parotid gland. This work has now been expanded in a study of the effects of sialographic procedures on the rat submandibular gland. The main duct is cannulated and under controlled flow, either water or lipid-soluble radiographic contrast medium is infused while monitoring the pressure changes in the system. Earlier work has shown characteristic pressure responses for each of the two contrast media, and that over a 3 to 4 minute infusion the radiographic appearance of the gland can be correlated with the pressure changes. The purpose of the present work is to correlate the pressure changes with the ultrastructural changes of the glandular parenchyma and the distribution of the contrast medium. We are currently working out various technical problems unique to this type of study, such as the loss of the contrast medium during routine processing of the tissues. Preliminary results indicate that with the lipid-soluble contrast medium there is considerable damage to the acinar and intralobular duct cells, while with the water soluble medium, to which HRP has been added as a tracer, there is less cellular damage but extensive leakage of the medium into the connective tissue spaces.

Significance to Dental Research:

These studies are expected to provide a better understanding of the structure and function of secretory cells. Secretory cells of the major and minor salivary glands provide the fluid environment of the oral cavity, and physiological or pathological changes in their function will greatly affect this environment. Although sialography is used as a clinical diagnostic procedure, virtually nothing is known of the effects of sialography on the salivary glands. Our studies should broaden the scientific basis of this procedure, potentially increasing its diagnostic utility and decreasing the possible damaging effects on the tissue.

Proposed Course:

During the coming year our continuing studies of the role of the Golgi apparatus and GERL in the transport and packaging of secretory material will be focused in the following areas: (1) radioautographic assessment of glycoprotein synthesis and transport in salivary and lacrimal acinar cells and pancreatic islet cells; (2) structural and cytochemical studies of alterations in the Golgi apparatus and GERL during secretory stimulation and resynthesis of granules; (3) correlation of morphological changes with cyclic nucleotide levels during secretion by the beige mouse lacrimal and parotid glands; and (4) characterization of Golgi-GERL relationships in parotid acinar cells of young rats. The sialographic studies will be continued, and if possible the effects of secretory stimulation on the response of the gland will be assessed.

It is anticipated that freeze-fracture studies of lacrimal and parotid acinar cells will be initiated to characterize membrane specializations related to (a) secretory granule discharge and luminal membrane endocytosis, (b) changes in intercellular junction structure during stimulated secretion, (c) differences between the membranes of the endoplasmic reticulum, Golgi saccules and GERL, and (d) sites of close contact of nerves with the acinar cell plasmalemma. Additionally, we plan to begin studies on the localization of peptide hormone and neurotransmitter receptor sites in salivary glands and other tissues, using EM radioautographic techniques.

2. Publications:

Hamosh, M., and Hand, A.R.: Development of secretory activity in serous cells of the rat tongue. Cytological differentiation and accumulation of lingual lipase. Dev. Biol. 65: 100-113, 1978.

Kelly, R.B., Oliver, C., and Hand, A.R.: The effects of vinblastine on acinar cells of the exorbital lacrimal gland of the rat. Cell Tiss. Res. 195: 227-237, 1978.

Bergeron, J.J.M., Sikstrom, R., Hand, A.R., and Posner, B.I.: Binding and uptake of ^{125}I-insulin into rat liver hepatocytes and endothelium. An in vivo radioautographic study. J. Cell Biol. 80: 427-443, 1979.

Oliver, C., Auth, R.E., and Hand, A.R.: Formation and fate of ethionine-induced cytoplasmic crystalloids in rat parotid acinar cells. Am. J. Anat. 155: 185-200, 1979.

Cournil, I., Leblond, C.P., Pomponio, J., Hand, A.R., Sederlof, L., and Martin, G.R.: Immunohistochemical localization of procollagens. I. Light microscopic distribution of procollagen I, III and IV antigenicity in the rat incisor tooth by the indirect peroxidase-anti-peroxidase method. J. Histochem. Cytochem. 27: 1059-1069, 1979.

Hand, A.R.: Synthesis of secretory and plasma membrane glycoproteins by striated duct cells of rat salivary glands as visualized by radioautography after ^{3}H-fucose injection. Anat. Rec., (in press).

Hand, A.R.: Cytochemical detection of peroxisomal oxidases. J. Histochem. Cytochem., (in press).

Hand, A.R.: Salivary Glands. In Bhaskar, S.N. (ed.): Orban's Oral Histology and Embryology, 9th Edition. St. Louis, C.V. Mosby Co., (in press).

Hand, A.R.: Cytochemical differentiation of the Golgi Apparatus from GERL. J. Histochem. Cytochem., (in press).

Broadwell, R.D., Oliver, C., and Brightman, M.W.: Neuronal transport of acid hydrolases and peroxidase within the lysosomal system of organelles: possible involvement of a specialized compartment of the agranular reticulum. J. Comp. Neurol. (in press).

Broadwell, R.D., Oliver, C., and Brightman, M.W.: Localization of neurophysin within organelles associated with protein synthesis and packaging in the hypothalamo-neurohypophysical system: an immunocytochemical study. Proc. Natl. Acad. Sci. USA, (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 DE 00163-03 LBS

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Ultrastructure and Biosynthesis of Cartilage Proteoglycans

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Hascall, Gretchen K.	Senior Staff Fellow	LBS NIDR
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	Lenk, Elaine	Visiting Associate	LBS NIDR
	Youmans, Patricia A.	Secretary (steno)	LBS NIDR
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	Floyd, Steven W.	Photographer	LBS NIDR

COOPERATING UNITS (if any)
Dr. Robert Cranley, St. Agnes Hospital, Baltimore, Md.; Dr. Bronwyn Bateman, Childrens' Hospital, Washington, D.C.; Drs. Martin, Brown, Pennypacker, and Kleinman, LDBA, NIDR; Dr. J. Kimura, LB, NIDR.

LAB/BRANCH
Laboratory of Biological Structure

SECTION
Experimental Morphology Section

INSTITUTE AND LOCATION
NIDR, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.03	0.73	0.30

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☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project uses electron microscopy, histochemistry, and autoradiography to study the biosynthesis of cartilage proteoglycans and their relationship to collagen in cartilage matrix. Current studies include electron microscopy of the Swarm rat chondrosarcoma and cells cultured from it, including the use of tracers to detect sites of uptake in the chondrocytes, and electron microscopy of abnormal cartilage from mice and humans.

PHS-6040
(Rev. 10-76)

1. Project Description:

Cartilage is a tissue in which cells, chondrocytes, synthesize and secrete proteoglycans and collagen to form an extensive extracellular matrix. The objectives of this project are: (1) to define the precise structure and localization of proteoglycans in the extracellular matrix; and (2) to determine how the chondrocyte synthesizes, packages, and secretes the proteoglycan and collagen for the matrix. Two experimental systems are utilized in this study: rat chondrosarcoma cells and abnormal cartilage from humans and mutant mice.

The Swarm rat chondrosarcoma forms a cartilage useful for model system research because the matrix is less dense than hyaline cartilage and allows better penetration of experimental reagents. In addition, these cells can be cultured so that they retain a chondrocyte appearance and rapidly synthesize new extracellular matrix (Dr. Vincent Hascall's laboratory, LB, NIDR, Z01 DE 00134-04). For these reasons I have chosen this system for extensive morphological studies on proteoglycan structure and biosynthesis.

Electron microscopy of the cells in the tumor, the cell pellet during isolation preparatory to culturing, and cultures after 18 hours show that all the cells are similar in structure to cartilage chondrocytes. The cytoplasm has large amounts of rough surfaced endoplasmic reticulum (RER) with distended cisternae filled with granular material, and an extensive Golgi apparatus, dispersed within the cytoplasm, associated with numerous condensing vacuoles and small smooth and coated vesicles. The cells show two unusual features: frequent close approaches of the RER to the cell surface, and short, wispy channels near the RER. Tracer experiments using ruthenium red as a cell surface and extracellular matrix granule label, and ferritin and colloidal thorium as extracellular space markers, are in progress to determine if these are pathways for uptake of materials from the matrix.

The extracellular matrix of the tumor resembles cartilage matrix, with matrix granules and collagen fibrils. During isolation of the cells the matrix is completely removed, but after 18 hours in culture the cells have formed a new matrix which resembles the tumor matrix except that the newly formed collagen fibrils lie close to the cell surface, often in deep invaginations of the cell surface. Experiments are planned using autoradiography of cultures pulse-labeled with ^{3}H-proline to determine if these invaginations are the site of collagen fibril growth.

Work has continued on the mutant mouse "Dmm" (disproportionate micromelia in an attempt to correlate short limbs with cartilage abnormalities. My studies on the morphology of the epiphyseal growth plate show a greatly distorted organization of cells in the columnar zone, but an extracellular matrix which appears nearly normal. Biochemical studies of this cartilage by members of LDBA, NIDR (Z01 DE 00201, 00202, 00203) indicate decreased amounts of proteoglycans and collagen, and possibly an increased water content. Further experiments to complete this project await successful breeding to produce more homozygous mutants.

Studies of the morphology of abnormal human cartilage have also been initiated. Dr. Bronwyn Bateman of Johns Hopkins University and Childrens' Hospital has access to surgical specimens, and in collaboration with Dr. George Martin (LDBA, NIDR, Z01 DE 00202-02) is performing biochemical and immunological studies on the samples. Ms. Elaine Lenk (LBS) and I are studying the ultrastructure of the tissues, in particular searching for any changes in the appearance and distribution of the matrix proteoglycans and collagen.

Proteoglycans probably play significant roles in controlling collagen fibril formation, mineral ion transport, and cartilage mineralization. These current studies are considered significant steps in a broader program aimed at elucidating mechanisms of matrix formation, composition and mineralization.

2. Publications:

Faltz, L.L., Reddi, A.H., Hascall, G.K., Martin, D.M., Pita, J., and Hascall, V.C.,: Characteristics of Proteoglycans Extracted from the Swarm Rat Chondrosarcoma with Associative Solvents. J. Biol. Chem. 254, 1375-1380, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 DE 00199-03 LBS

PERIOD COVERED

October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

In Vitro Studies of Secretory Cell Structure and Function.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Oliver, Constance	Senior Staff Fellow	LBS	NIDR
OTHER:	Hand, Arthur R.	Chief, LBS	LBS	NIDR
	Lenk, Elaine V.	Visiting Associate	LBS	NIDR
	Waters, Judith F.	Biologist	LBS	NIDR
	Worrell, Dorrette F.	Biologist	LBS	NIDR
	Weatherall, Cecilia C.	Bio. Aid (Biochem.)	LBS	NIDR
	Youmans, Patricia A.	Secretary (steno)	LBS	NIDR
	DeGraff, Barbara A.	Clerk-typing	LBS	NIDR
	Floyd, Steven W.	Photographer	LBS	NIDR
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COOPERATING UNITS (if any)

J. Greenberg, LDBA

LAB/BRANCH

Laboratory of Biological Structure

SECTION

Experimental Morphology Section

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NIDR, NIH, Bethesda, Md. 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.73	0.70	2.03

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Secretory processes in several cell types are currently under investigation. Cell dissociation and short term culture (up to 1 month) methods have been established for rat exorbital lacrimal, parotid and pancreatic acinar cells. These cultures are being used to study various aspects of the secretory process. Initial emphasis is being placed on morphological, cytochemical and biochemical characterization of the cultured cells. Additionally, uptake of horseradish peroxidase and internalization of ^{125}I labeled cell membrane are being investigated, in vivo and in vitro. The events involved in matrix deposition by cultured odontoblasts, and melanosome formation in cultured neural crest cells are also being studied.

PHS-6040
(Rev. 10-76)

1. Project Description

The primary objectives of this investigation are to establish procedures for short term culture of isolated exocrine gland acinar cells and to utilize these cultures in investigations of events involved in the secretory process.

Methods Employed:

The isolation of the acinar cells involves sequential treatment of the glands with EDTA and collagenase-hyaluronidase solutions. The dissociated cells are filtered through 500 μ and 25 μ Nytex filters and the acinar cells separated on a Ficoll gradient. The cells are cultured as suspension cultures at 35°C in a humidified atmosphere of 5% CO_2 in air in Ham's nutrient mixture F-12 supplemented with antibiotics and the appropriate secretagogue (exorbital lacrimal, 10^{-6}M carbamyl choline; pancreas 10^{-5}M carbamyl choline; parotid 10^{-6}M isoproterenol). Protein synthesis was assessed by determining the amount of ^{3}H-leucine incorporated into TCA precipitable protein.

Membrane reutilization was studied both *in vivo* and *in vitro*. For *in vivo* studies, horseradish peroxidase (HRP) (1 mg/gm body weight) was injected into the saphenous vein of adult, male NIH Swiss mice or Wistar-Furth rats. Some animals received secretagogue (isoproterenol, 20 mg/kg body weight; pilocarpine 40 mg/kg body weight) prior to or concomitant with the HRP injection. Animals were sacrificed by vascular perfusion of fixative, at varying times following HRP administration. The glands were excised, and sections incubated for peroxidase activity, or for demonstration of various marker enzymes, i.e. thiamine pyrophosphatase for Golgi saccules and trimetaphosphatase and acid phosphatase for lysosomes. For *in vitro* studies of membrane reutilization isolated acini were prepared by collagenase-hyaluronidase digestion, filtered through Nytex filters and separated on a ficoll gradient. Suspensions of acini were iodinated enzymatically utilizing a lactoperoxidase glucose oxidase system. Internalization and fate of ^{125}I labeled membrane was followed by light and electron microscope radioautography. Other acini were cultured in medium containing 1% HRP as a soluble phase marker.

Major Findings:

Dissociation of rat exorbital lacrimal glands by alternate incubations in EDTA and collagenase-hyaluronidase solutions yielded a cell suspension which was approximately 90% acinar cells and had a viability of about 80% as determined by trypan blue exclusion. After isolation the single acinar cells rapidly reaggregated to form small acini of 5 - 20 cells. The cells retained their original polarity upon reaggregation. When these isolated cells were cultured, they maintained good morphology for approximately 24 hours. After 48 hours, the apical portions of the cells became filled with secretory granules; lipid and lysosomes accumulated in the basal cytoplasm. Addition of secretagogue to medium prevented these changes and allowed maintenance of the cells for up to 1 month. Measurements of protein synthesis showed that after 10 days in culture, the cells incorporated ^{3}H-leucine at the same rate as freshly isolated cells. Additionally, exorbital lacrimal gland acinar cells cultured for up to one week still retained cytochemically demonstrable peroxidase activity, and responded to stimulation with carbamyl choline. These findings indicate that exocrine gland acinar cells may be maintained in vitro and utilized as a model system for the study of secretory processes.

Initial in vitro studies on membrane reutilization in isolated pancreatic acinar cells showed that HRP was taken up from the cell surface in small endocytic vesicles and transported to lysosomes. No HRP reaction product could be found in Golgi saccules, GERL, or immature or mature secretory granules. On closer examination, the endocytic organelles proved to be of a morphological type not previously noted in in vivo studies on the uptake of HRP from the apical surface of parotid acinar cells. In the in vitro studies, since the HRP was in the medium and had access to all surfaces of the cell, it was not always possible to distinguish endocytic structures which originated from the apical cell surface from those which originated from the lateral and basal surfaces. In vivo junctional complexes at the apices of the cells restrict the passage of large molecules, such as HRP, between cells. Therefore intravenously (IV) administered tracers normally cannot gain access to the acinar lumen and are endocytosed only by the lateral and basal cell surfaces. In resting and stimulated mouse and rat exorbital lacrimal, parotid and pancreatic acinar cells, IV administered HRP was localized primarily in endocytic vesicles, elongated cisternae and secondary lysosomes. The uptake of HRP into these cisternae is unique to IV injection of tracer. Enzyme cytochemical characterization of these cisternae showed that they were a previously unrecognized component of the lysosomal system located adjacent to the basal and lateral cell surfaces. These basal lysosomes possessed trimetaphosphatase activity, but lacked acid phosphatase activity. It thus appears that exogenous material and cell membrane from the lateral and basal cell surfaces are endocytosed in distinct organelles and may be directed, at least initially, to a separate component of the lysosomal system.

Mechanisms underlying secretion of dentin matrix by cultured odontoblasts are being studied by Mrs. Dorrette Worrell. Tooth buds, removed from 1 day old Sprague-Dawley rats, are cultured for up to 7 days. The tooth buds are grown on nucleopore filters in organ culture dishes with BGJb medium supplemented with penicillin, vitamin C and horse serum. Matrix deposition was apparent by 2 days _in vitro_. The role of the Golgi apparatus and GERL in the synthesis and packaging of matrix proteins is currently under investigation. The effect of microtubular inhibitors on this system will be studied.

Many of the steps involved in melanosome formation in pigmented cells parallel those of secretory granule formation. Understanding events controlling melanosome production may yield new insights into the process of secretory granule production. Cultured neural crest cells grown in the presence of fetal bovine serum (FBS) rapidly differentiate into melanocytes, while those grown with horse serum become neuron-like. The presence of DMSO in the medium with FBS reversibly inhibits pigmentation in these cells, although the cells do contain premelanosomes. Cells grown in FBS with and without DMSO will be examined at intervals after exposure to and removal of the drug. The effects of the various treatments on the formation of melanosomes will be investigated using electron microscopy and enzyme cytochemical localization of tyrosinase (a marker enzyme for melanin synthesis). These studies are being carried out in collaboration with Dr. Judy Greenberg, LDBA, NIDR.

Significance to Dental Research:

In vitro studies of exocrine gland acinar cells should provide a greater understanding of both the controlling mechanisms and synthetic pathways involved in their secretory processes. Salivary gland secretions are absolutely essential for maintainance of the health of the oral cavity. Therefore, knowledge of the normal secretory process is critical to our understanding of many pathological conditions which affect dental health.

Proposed Course of Project:

Cultured exocrine cells will be further characterized with respect to their cellular morphology and cytochemistry, protein synthetic capacity, and response to secretagogues. Additional studies will examine the distribution of membrane receptors, membrane reutilization, and the role of microtubules and microfilaments in the secretory process.

2. Publications:

Oliver, C., 1979. Isolation and maintenance of differentiated exocrine gland acinar cells in vitro. _In Vitro_ (in press).

Oliver, C., 1979. Cytochemical localization of acid phosphatase and trimetaphosphatase activities in exocrine acinar cells. J. Histochem. Cytochem. (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 DE 00012-17 LBS

PERIOD COVERED

October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Infrared and Raman Spectroscopic Studies of Teeth and Bones and Related Synthetic Compounds

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Fowler, Bruce O.	Research Chemist	LBS NIDR
OTHER:	Youmans, Patricia A.	Secretary (steno)	LBS NIDR
	DeGraff, Barbara A.	Clerk-typing	LBS NIDR

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Biological Structure

SECTION

Molecular Structure Section

INSTITUTE AND LOCATION

NIDR, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.1	1.0	0.1

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The main objective is to determine compositional and structural details of the inorganic phase in teeth and bones. Infrared and Raman spectroscopy as well as chemical methods are employed in these studies. Methods are devised for the preparation of synthetic calcium apatites having controlled physical properties (crystal size and perfection) and chemical constituents (e.g., hydroxide, fluoride, chloride, carbonate, water and acid phosphate). The vibrational spectra of these apatites and related compounds are assigned and characterized. Isotopically enriched apatite analogs are prepared to facilitate spectral assignments. The spectroscopic assignments and supplemental spectral data (temperature dependency and polarization) are then utilized to establish compositional and structural details of the apatites in question which include: the type and geometry of constituent ions; the site or number of sites occupied by the ions; orientation of ions; chemical bonding and interactions of ions; and semi-quantitative estimations of the constituents present. The results for these controlled apatite systems are then related to the inorganic phase in calcified tissues.

PHS-6040
(Rev. 10-76)

1. Project Description:

Objectives:

The main objectives are to determine compositional and structural details of the inorganic phases in teeth and bones, with special emphasis on normal, abnormal, carious and chemically-treated human tooth enamel.

Methods and Approaches Employed:

Infrared and Raman spectroscopy and chemical methods are the primary tools of these studies. An understanding of the infrared and Raman vibrational spectra of various synthetic apatites and related compounds is necessary to determine corollary compositional and structural details for the inorganic phase(s) of hard tissue. Hence, these studies entail identification of the vibrational origin of infrared and Raman spectral bands for pure hydroxy-, fluor-, and chlorapatite, for mixed apatites containing hydroxide, fluoride, chloride, carbonate, acid phosphate, water and different cations, and for related calcium phosphates. The combined spectral data are then utilized to establish compositional and structural details of these apatites. These include the type of geometry of ions, orientation of ions, chemical bonding and interactions of ions, and semi-quantitative estimations of constituents present. Specialized spectroscopic techniques involving reflectance, polarization, low and high temperature, and high pressure devices are utilized in obtaining spectra. Methods are developed for the synthesis and purification of the compounds studied that require design and construction of specialized apparatus to maintain the rigid experimental conditions (e.g., high temperature and pressure) required to form apatites of controlled chemical and physical properties. Isotopically substituted analogs are prepared to facilitate assignment of spectral bands. The techniques are supplemented by chemical analyses to ascertain purity and chemical composition of the preparations.

Major Findings:

The proposed course of this year's project, continuation of the carbonate apatite study, was temporarily altered to include development and evaluation of infrared and Raman spectroscopic methods for quantitative determination of hydroxide ion contents in synthetic apatites of known chemical composition. Both synthetic and biological apatites have been indicated to contain variable amounts of hydroxide ions based on spectroscopic and other methods but quantitation has been, in general, uncertain. Quantitation of hydroxide ions in synthetic and biological apatites is important in order to better characterize details of apatite composition and structure. Dr. J.L. Meyer of this Laboratory has developed a method for chemically determining hydroxide ion contents in apatites and has prepared a series of apatites containing varying hydroxide contents. These Meyer apatites had chemically determined hydroxide contents of 0 to 60% of the theoretical based on $1OH/3PO_4$ as 100% hydroxylated, Ca/P molar ratios of 1.39 to 1.60, HPO_4 contents of 1 to 19% of the total PO_4, CO_3 contents of 0.1 to 1.5 wt. % and H_2O contents of 9 to 12 wt. %. These nineteen Meyer apatites of known hydroxide contents along with five high purity hydroxyapatite standards (Fowler), containing about 95% of the theoretical hydroxide by chemical analyses, were used in this study. The basic method used for infrared and Raman spectroscopic quantitation was as follows: (1) OH to PO_4 band area ratios were established for the 95% hydroxide containing hydroxyapatite standards, (2) a linear decrease in OH/PO_4 ratios to zero as a function of decreasing hydroxide content was assumed, and (3) hydroxide contents of the Meyer apatites were computed from their respective OH/PO_4 ratios on the basis of the standards. The hydroxide stretching band was used for both infrared and Raman measurements. The ν_2 PO_4 band was used for Raman measurements; the ν_4 PO_4 band, corrected for the hydroxide librational band interference, was used for infrared measurements.

Infrared Measurements: Important sample preparation factors and variables to consider in obtaining infrared spectra of powders in pressed alkali halide pellets or mulls for quantitative measurements are: (1) sample particle size consistency, (2) sample particle shape, (3) homogeneous dispersion of sample particles in pellet, (4) Christiansen effect, (5) pressure induced sample changes and sample interactions with pellet or mull material, and (6) differences between index of refraction of sample and pellet or mull material. All samples were reduced to a consistent small particle size, about 2 to 4 microns, by standardized gentle grinding in the dry state; adverse effects caused by the Christiansen effect, band asymmetry, were reduced at this particle size level but not completely in the hydroxide stretching region. Effects of factor (5) were found to be minimal by comparison with spectra obtained from a different sampling technique, vaseline mulls. Adverse effects of factor (3) and, in part, factor (1) were reduced by the OH/PO_4 band area ratio method employed; poor sample dispersion, resulting in an apparent lower sample concentration, was essentially compensated for by using the ratio method. In addition, the OH/PO_4 ratio method is essentially independent of differences in water and carbonate contents of the apatites compared whereas with infrared measurements based on sample concentration, sample weight corrections for extraneous constituents are necessary. The indexes of refraction of potassium bromide, vaseline and the apatites studied are about 1.56, 1.48 and 1.60 to 1.64, respectively. Slight asymmetry of the hydroxide stretching band, causing quantitation uncertainty, was observed in vaseline mull spectra of the hydroxyapatite standards. This band asymmetry was minimized by using mulling fluids with indexes of refraction of about 1.60 to 1.64. However, additional requirements for an acceptable mulling fluid are high viscosity, lack of interfering bands in regions of interest and inertness. A preliminary search did not reveal a fluid fulfilling all these requirements, and vaseline, overall, was more suitable. The potassium bromide index of refraction is lower than ideal, but it was used because of its suitable characteristics as a pellet material. Crystal shape effects on infrared band areas and intensities were not evaluated in this study. The reproducibilities of hydroxide contents determined from duplicate potassium bromide pellets and duplicate vaseline mulls were ± 5% relative or less. The hydroxide contents of the twenty-one apatite samples derived from infrared OH/PO_4 band area measurements obtained from potassium bromide pellet and vaseline mull spectra agreed within (-11 to + 18, maximum; -7 to +9, average) absolute percentage units from the chemically determined hydroxide contents. The hydroxide contents derived from OH/PO_4 band intensity ratios deviated more (-31 to +6, maximum; -12 to +4, average) from the chemical values than those derived from area ratios. Band intensity measurements do not account for broadening of spectral bands which can be caused by numerous factors in these apatites whereas band broadening is accounted for in band area measurements, and closer agreement with the chemical values, as expected, was observed. The OH/PO_4 band area ratios for the monoclinic and hexagonal hydroxyapatite standards agree within about ± 10%; hence, monoclinic hydroxyapatite can be used as an hydroxide standard for the hexagonal apatites using infrared OH/PO_4 area ratios. Final measurements for differences in OH/PO_4 band intensity ratios between monoclinic and hexagonal hydroxyapatite are incomplete. These results show that the infrared method as described herein is capable of semi-quantitative determination of hydroxide contents in apatite of compositions indicated. Spectroscopic hydroxide quantitation in apatites containing higher carbonate contents, additional cations, and especially fluoride and chloride anions, which can cause hydroxide band shifts, area and intensity changes, will require additional evaluation.

Raman Measurements: The main experimental problems and factors to overcome or evaluate in obtaining Raman spectra for quantitative measurements are: (1) sample changes, decomposition and band broadening caused by laser beam heating, (2) high spectral background arising from fluorescence, (3) crystal orientation effects on band areas and intensities, and (4) possible crystal size and shape effects on band areas and intensities. To reduce localized laser beam heating effects and to increase Raman signal, the apatite powders were pressed under high pressure into cups in aluminum disks. The compacted apatite particles and their intimate contact with the aluminum facilitated heat transfer and reduced localized heating. Laser light power was adjusted to levels (∿ 150 mw at 4880 A) that caused no visually detectable sample changes or discoloration, and spectra at different exposure times of laser irradiance were compared for thermally induced changes. Spectra recorded after 2 and 20 hours laser irradiance at 150 mw were essentially the same; however, instantaneous sample changes were not entirely excluded. Background fluorescence was reduced to acceptable levels by aging the samples for 4 to 16 hours in the laser beam. Band intensity variations arising from crystal orientation was minimal because of their small crystal size and their random orientation in the compressed powders. Also, instrumental polarization checks on the larger size (∿ 15 microns) hydroxyapatite standards did not show crystal orientation effects. Possible spectral effects arising from crystal size and shape have not been fully evaluated. Hydroxide contents of twenty-four apatites were obtained from OH/PO_4 band area and band intensity ratios. The reproducibility of duplicate runs was about ± 5% relative. A correlation between chemically determined and Raman spectroscopically deduced hydroxide contents exists; however, deviations from the chemical values were, overall, greater than those determined by infrared analyses. The hydroxyapatite standards, containing about 95% of the theoretical hydroxide, showed Raman spectroscopically deduced hydroxide contents ranging from 80 to 120% by OH/PO_4 band area ratio measurements. Efforts to reduce and/or understand this deviation are underway. Attempts to evaluate the effects of particle size from spectra of 10 micron and larger size hydroxyapatite crystals ground to smaller size were not entirely successful. The ground smaller size crystals had a higher interfering spectral background arising from increased Rayleigh scattering, and other probable sources including impurities and numerous nascent crystal surfaces introduced by grinding. Monoclinic hydroxyapatite was not found to be a suitable Raman standard for hydroxide measurements in hexagonal apatites based on OH/PO_4 band intensity ratios. The Raman hydroxide stretching band intensity of monoclinic hydroxyapatite is about twice that of the hexagonal form whereas the hydroxide band areas for both forms agree within about ± 20%. The markedly greater Raman hydroxide stretching band intensity of monoclinic hydroxyapatite probably arises from its ordered hydroxide ion arrangements; hydroxide ions are disordered in the hexagonal form. Possible quantitation of monoclinic and hexagonal domains in hydroxapatite crystals may be possible based on the normalized intensity, width and area of the Raman hydroxide stretching band.

Significance to Dental Research:

Characterization and assignment of the infrared and Raman bands of apatite containing biologically relevant ions and those of related calcium phosphates are essential in establishing corollary structural details for the inorganic phases of teeth and bones. The types and degree of incorporation of hydroxide, fluoride, chloride and carbonate ions, water, acid phosphate and different cations into biological apatites have bearing on the chemical, physical and biological properties of hard tissue.

Proposed Course of Project:

The infrared and Raman spectroscopic study for semi-quantitative determinations of hydroxide ions in apatites will be completed and prepared for publication.

The preparation and characterization of specific biologically relevant synthetic carbonate apatites will be continued. Results will be related, where applicable, to the carbonate components in tooth enamel.

Raman single-crystal symmetry species data on fluorapatite and hexagonal hydroxyapatite and both Raman and infrared powder data on fluorapatite, hexagonal and monoclinic hydroxyapatite, plus additional Raman data to be collected on hydroxide librational modes, will be prepared for publication.

Further work will continue on infrared and Raman external mode band assignments for fluorapatite and both the hexagonal and monoclinic forms of hydroxyapatite and chlorapatite using combined data from band symmetry species, isotopic band shifts, band temperature dependency, band intensity and mixed OH, F, Cl apatites.

2. Publications:

Fowler, B.O.: Infrared spectra of apatites. In Brown, W.E. and Young, R.A. (eds): Internat. Symposium on Structural Properties of Hydroxyapatite and Related Compounds. Gaithersburg 1968. New York; W.A. Benjamin (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 DE 00074-07 LBS

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Protein-Crystal Relationships in Mineralized Tissues

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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LAB/BRANCH
Laboratory of Biological Structure

SECTION
Molecular Structure Section

INSTITUTE AND LOCATION
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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5.76	4.26	1.50

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The biochemical and biophysical properties of developing skeletal and dental tissue proteins are being studied by several techniques. Dentin, bone and enamel matrix proteins are also investigated as to their functional and structural influences on active mineralization in biological systems. Special emphasis is placed on phosphoprotein and glycoprotein biochemistry in these hard tissue matrix studies.

1. Project Description:

The long range goals of this research project are twofold: (1) to study the structural and chemical properties of the inorganic and protein constituents of bones and teeth; and (2) to examine critically the changes that occur in these components during development, growth and disease states. Many of our procedures employ both model and *in vitro* systems as well as isolated biological entities. It is our hope that this multifaceted approach will provide a more thorough understanding of the biological mineralization process.

In the past two fiscal years, a sequential dissociative extraction scheme was devised to differentially isolate fetal tooth matrix proteins. In this scheme, great care was taken to avoid proteolysis at all phases of the overall extraction process in order to obtain the fetal dentin and enamel matrix proteins in as native and non-degraded a form as possible. Further, the extraction scheme was specifically designed to enrich those tissue proteins most closely associated with enamel and/or dentin apatite crystallites into a separate protein fraction. The details of these extraction procedures and the electrophoretic procedures devised to follow them were described in prior yearly reports. These efforts had allowed us to isolate three separate protein fractions from fetal bovine teeth: (1) the amelogenins, a series of proteins present in fetal but not fully formed enamel that are very rich in proline (25-30%), glutamic acid (17-20%), leucine (10-12%) and histidine (6-8%); (2) the apatite-associated fetal enamelins, a series of acidic enamel proteins, rich in aspartic and glutamic acid (27-30%), proline (14-16%), glycine (10-12%) and serine/threonine (13-15%); and (3) the dentin phosphoproteins, extremely rich in aspartic acid (30-40%) and serine/phosphoserine (30-40%).

The fetal enamelins have an overall amino acid composition which suggests that they are the most probable precursors of those proteins that survive amelogenesis to persist in the final, fully erupted tooth enamel matrix. This suggestion is supported by two additional findings obtained this year. First, the fetal enamelins have an extremely high affinity for apatite crystals and are selectively adsorbed by synthetic apatite from mixtures of total fetal enamel proteins (enamelins plus amelogenins) even in 4 molar guanidine, an extremely potent dissociative agent. Second, a higher proportion of fetal enamelins (relative to amelogenins) can be extracted from fetal calf teeth relatively late in development (7-8 months *in utero*) than from somewhat younger ones (5-6 months *in utero*). Furthermore, careful analysis of the total quantities of enamelins extracted from developing bovine teeth of different fetal ages suggests that these proteins are synthesized at all stages of enamel development.

Last year, we reported that the fetal enamelins contained three major species on SDS-urea P.A.G.E. at molecular weights of ca. 72,000, 56,000 and 49,000 and that these three species showed ideality vs. known m.w. standards in Ferguson plots from SDS-urea gels of differing polyacrylamide concentrations. We have now extended these electrophoretic analyses to two other SDS-P.A.G.E. systems not containing urea. Ferguson ideality was achieved vs. known markers for the three major fetal enamelins in each of these additional gel systems, thus indicating that the molecular weights determined for them by the SDS-electrophoretic method were correct. These three proteins, which migrate together on ion-exchange chromatography, stain positively on polyacrylamide gels both for organic phosphate and/or sialic acid (Stains - All) and for carbohydrate (PAS stain). On gel filtration chromatography in 4 molar guanidine on either Sepharose CL-6B or Sephacryl S-200, the fetal enamelins also contained three major components at molecular weights (vs. known m.w. standards) of ca. 72,000, 55,000 and 48,000 and relatively small amounts of lower m.w. components at about 25,000, 15,000 and 10,000 Daltons. These lower m.w. fetal enamelin components had a combined amino acid composition almost identical to that obtained for the higher m.w. fetal enamelin triplet, thus suggesting that they may be proteolytic breakdown products of the larger species. Finally, we have determined chemically that the high molecular weight fetal enamelin triplet proteins contain approximately 0.2% organic phosphorus, 4-6% sialic acid, 4-5% hexosamine (with a glucosamine/galactosamine ratio of 3/1) and ca. 20% total carbohydrate (anthrone method). Thus, these high molecular weight, acidic fetal enamel proteins, described by us for the first time, are phosphorylated, sialo-glycoproteins.

As mentioned above, the amelogenins are only found in developing enamel, being absent from fully erupted teeth. Last year, we reported that the bovine fetal amelogenins ranged in molecular weight from 40,000 - 10,000 on SDS - urea P.A.G.E. and gave linear but non-ideal Ferguson plots vs. known m.w. standards. We have now extended these electrophoretic analyses and can confirm that these proline-rich fetal enamel proteins show non-ideal SDS electrophoretic behavior regardless of the gel system utilized. When urea is omitted from the SDS polyacrylamide gels, the fetal bovine amelogenins appear lower in molecular weight, ranging from ca. 25,000 - 8,000 Daltons. Thus, it is impossible to ascertain correct molecular weights for this particular set of fetal enamel proteins by electrophoretic methods. This is probably related to conformational and/or SDS-binding anomalies associated with their unusually high (25-30%) proline contents.

As previously found by others, we observed that the amelogenins decrease in absolute amounts isolated from teeth of increasing degree of mineralization and progressing enamel development. We have also found that the fetal bovine amelogenins consist of from 8 to 10 species on gel filtration chromatography in 4M guanidine on Sephacryl S-200. These species range in molecular weight from ca. 28,000 - 6,000 vs. known m.w. markers by this method of analysis. Moreover, the population of amelogenins shifts from one dominated by the higher molecular weight components of the series in enamel from younger fetuses (3-4 mos. *in utero*) to a population dominated by lower m.w. members in enamel from older fetuses (7-8 mos. *in utero*). This high to low m.w. population shift was also observed in comparisons of less well-mineralized with more fully-mineralized enamel tissue within any given fetal age group. Thus, it would appear that the amelogenins are gradually converted to lower and lower m.w. components prior to their ultimate removal with increasing mineralization in the normal enamel maturation process. It was interesting to note that the smaller the molecular size of our amelogenin components, the higher was their relative organic phosphate content. This suggests that the previously described phosphopeptides of fetal enamel most probably result from the breakdown of higher m.w. amelogenin proteins. Finally we have determined chemically that the fetal bovine amelogenins, as a class, are also glycoproteins, containing about 1% each of sialic acid and hexosamine and ca. 10-15% total carbohydrate (anthrone method), data consistent with our observation that these proteins stain positively with PAS after electrophoresis on polyacrylamide gels. This fact, heretofore unreported, taken together with their odd SDS-P.A.G.E. behavior, their highly unusual amino acid composition and their rather transient appearance in enamel formation makes the amelogenins a challenge to all concerned with the molecular aspects of the tooth formation process.

Last year, we reported the identification of at least three dentin-specific proteins on SDS-urea P.A.G.E. We have recently extended this work using dissociative ion exchange (7M urea, DEAE-cellulose or DEAE-Sephacel) and gel filtration (4M guanidine, CL-6B Sepharose) chromatography. We can now report that aside from a major phosphoprotein, fetal calf dentin also contains at least three phosphorylated glycoproteins in the 30,000 - 80,000 m.w. range and ca. 2-3 phosphorus-containing glycoproteins in the 10,000 - 25,000 m.w. range. The structure and function of these proteins as well as their relationship to the major phosphoprotein of dentin are completely unknown at this time. We have also purified our fetal calf dentin phosphoprotein further by a series of ion-exchange and dissociative gel filtration chromatography steps. This purified protein, which chromatographs as a single symmetric peak on CL-6B Sepharose (4M guanidine) and appears as a single band both on SDS-P.A.G.E. (± urea) and on urea-P.A.G.E. (without SDS), comprises from one-half to two-thirds of the total fetal calf dentin noncollagenous protein matrix. Its molecular weight on both SDS electrophoretic gels and gel filtration chromatography under dissociative conditions is ca. 96,000. It contains 6-7% organic phosphorus, 1% each of sialic acid and hexosamine (with a glucosamine/galactosamine ratio of 1/3), and ca. 5-6% total carbohydrate (anthrone method). Its most abundant amino acids were serine/phosphoserine (42%), aspartic acid (38%), lysine (5.5%), glycine (4.5%) and glutamic acid (3.5%). This preparation is much higher in molecular weight and more abundant in non-serine/aspartic acid components than any other heretofore isolated. Preliminary work with rodent dentin phosphoproteins (rat incisor, courtesy of Dr. William T. Butler, University of Alabama in Birmingham, and hamster molar) isolated using our dissociative, sequential extraction scheme indicates that the apparently high molecular weight we observed for the fetal calf dentin phosphoprotein results, at least in part, from species differences. Our preliminary data on the rodent dentin phosphoprotein indicates a molecular weight of only 70,000 - 75,000 for this species.

In prior years, we had reported that dentin phosphoproteins inhibit apatite formation from an amorphous calcium phosphate precursor in synthetic extracellular fluids. We now report that this inhibition activity is markedly affected by pretreatment of the phosphoprotein with ionic calcium and/or collagen fibrils. Significantly diminished inhibition of de novo apatite formation was observed after prior exposure of a given phosphoprotein preparation (egg yolk phosvitin, a model phosphoprotein utilized to set boundary conditions for these experiments, fetal calf dentin phosphoprotein and rat incisor dentin phosphoprotein) to (1) either ionic calcium or reconstituted rat tail tendon collagen fibrils alone and (2) combinations of these two interfering agents. In all cases, the combination experiment yielded a greater diminution of inhibition activity than when either interfering agent was used individually. Inasmuch as the bulk of dentin mineralization may well involve apatite formation from pre-existing crystal seeds, we have extended these observations of phosphoprotein effects on apatite formation using seeded crystal growth systems in a collaboration with Dr. E. D. Eanes of this Laboratory. All three of the above phosphoprotein preparations provided two stages of inhibition of apatite crystal growth/replication in seeded, metastable synthetic solutions. Pretreatment of the crystal seeds with phosphoprotein induced an initial lag phase in the crystal growth process, probably associated with seed surface phenomena, and then prolonged the subsequent reaction period where octacalcium phosphate is the predominant new mineral phase prior to its eventual conversion to the final, induced apatite product. A combination of seed pretreatment with phosphoprotein together with direct addition of soluble phosphoprotein (17 μg/ml) to the metastable reaction solution completely blocked the crystal growth process at the initial lag phase step. In contrast to the results obtained in our de novo apatite formation system, reconstituted collagen fibrils had no effect on phosphoprotein inhibition of apatite crystal growth in seeded systems. On the other hand, however, pretreatment of the phosphoproteins with calcium ions (final Ca^{+2} concentration, ca. 20 μM) did diminish their inhibition activity toward seeded apatite crystal growth.

In a companion study conducted in collaboration with Dr. R. A. Gelman of the Laboratory of Biochemistry, NIDR, we examined the effect of these three phosphoprotein preparations on collagen fibrillogenesis in vitro. In each case, the addition of soluble phosphoprotein inhibited collagen fibril formation to a significant degree. Once formed, however, the collagen fibrils were identical to controls formed in the absence of phosphoprotein, when viewed in the electron microscope. In contrast to the results on apatite formation, pretreatment of the interfering phosphoproteins with ionic calcium (final Ca^{+2} concentration, ca. 20 μM) greatly increased their inhibitory activity towards collagen fibrillogenesis. When all these in vitro results on phosphoprotein effects on apatite formation and collagen fibrillogenesis are taken together, a strong picture develops for a potential role of the dentin phosphoprotein as a principal regulatory agent in both dentinogenesis and dentin mineralization. Moreover, the degree of activity for the dentin phosphoprotein in these two processes appears to be capable of modulation by calcium ions. Thus, the importance of this unusual phosphoprotein to the formation and structural integrity of dentin tissue is strongly suggested from these experiments.

Considerable effort will be spent in the future to continue these biochemical and biophysical studies of tooth matrix proteins. We have made significant progress to date in biochemically characterizing the fetal calf enamel proteins, but need to purify the higher molecular weight fetal enamelin and amelogenin components further to better understand their structure and function. Careful comparisons at the peptide level between high and low molecular weight components within each class should yield clues as to their eventual fate in the normal enamel maturation process. Once these detailed molecular investigations have reached this stage of development, attention can be paid to the structural and biophysical properties of the fetal enamel proteins, especially with regard to how they may affect the enamel mineralization process. Similarly, once a high enough degree of protein purity has been attained, immunological methods can be employed to augment these efforts. We have initiated preliminary biochemical studies of the human adult enamel proteins in order to find out just which molecular species survive the post-eruptive enamel maturation process and to better understand their structure and function. These efforts will be complementary to our ongoing studies of the fetal enamel proteins and should enhance our overall program in enamel protein chemistry. At the other end of the developmental scale, we have initiated preliminary studies of neonatal hamster molars with an eye toward investigating the biosynthesis of enamel matrix proteins in vitro. We have found so far that there are many similarities between the different enamel protein fractions of the 2-6 day old unerupted hamster and fetal calf molars. Further, following techniques developed by Dr. Lyaruu in Amsterdam, we have established that hamster molar tooth germs can be placed in short term (2-4 day) organ culture both prior and subsequent to the onset of normal enamel matrix production and mineralization. These studies will be expanded this coming year to investigate the biogenesis and fate of the different developing enamel matrix fractions. Finally, we will continue our studies, of the dentin phosphoproteins by focusing on both their intrinsic biochemical and biophysical properties and their precise mode of action in dentinogenesis and mineralization.

Skeletal and soft tissue calcification diseases cut across a wide swath of medical and dental problems. Orthopedic diseases, arthritis, atherosclerosis, certain renal and endocrine disorders, for example, all involve the structure and metabolism of the skeleton. Still further, is the need to understand thoroughly both the development and structure of mineralized tissues basic to the caries, periodontal disease and craniofacial anomalies research objectives of the NIDR. The bones and teeth are composite tissues consisting of cellular, collagenous, glycoprotein, phosphoprotein and inorganic components. As a consequence, their physiology and pathology are considerably more complicated than those of other tissues in the body. This research program is designed to study in detail the relationships that exist between the various constituents of hard tissues both from structural and functional points of view.

2. Publications

Editor: Nylen, M.U. and Termine, J.D.: Proceedings, Third International Symposium on Tooth Enamel. Washington, IADR/AADR, J. Dent. Res. 58, Spec. Iss. B, 1979, 358 pp.

Termine, J.D., Torchia, D.A. and Conn, K.M.: Enamel Matrix: Structural Proteins. In: Nylen, M.U. and Termine, J.D. (Eds.): Proceedings, Third International Symposium on Tooth Enamel. Washington, IADR/AADR, J. Dent. Res. 58, Spec. Iss. B, 1979, pp 773-780.

Peckauskas, R.A., Termine, J.D., and Pullman, I.: ESR studies on tooth phosphoproteins using manganese ions as probes. Arch. Biochem. Biophys. 193: 186-190, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 DE 00088-06 LBS

PERIOD COVERED

October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Chemical, Structural, and Morphological Studies on Calcium Phosphates.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Eanes, Edward D.	Chief, Molecular Structure Section	LBS NIDR
OTHERS:	Martin, Garland N. Jr.	Research Chemist	LBS NIDR
	Hailer, Arthur	Chemist	LBS NIDR
	Rattner, Steven L.	Chemist	LBS NIDR
	Youmans, Patricia A.	Secretary (steno)	LBS NIDR
	DeGraff, Barbara A.	Clerk-typing	LBS NIDR
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COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Biological Structure

SECTION

Molecular Structure Section

INSTITUTE AND LOCATION

NIDR, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 3.02	PROFESSIONAL: 1.0	OTHER: 2.02

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The formation and maturation of calcium phosphates prepared from physiological-like solutions is being studied with a variety of ultrastructural and physical-chemical techniques such as electron microscopy, x-ray diffraction, B-E-T surface area methods, and standard analytical chemistry procedures. Topics of current interest include (1) the effect of ionic fluoride, magnesium, and carbonate on physical properties of crystalline apatite such as crystallinity, specific surface area, and particle morphology, and (2) the effect of these ions on the formation, growth, and stability of precursors to the apatite phase.

PHS-6040
(Rev. 10-76)

1. Project Description:

Objectives:

The purpose of this project is to study both the preparation from solution and the physico-chemical/ultrastructural properties of calcium phosphate salts, with particular emphasis on the formation and maturation of apatite from physiological-like solutions. The principal objective is to acquire a more complete understanding of biomineralization processes, with special emphasis on the physical-chemical aspects of mineral deposition in vertebrate hard tissue. During the year covered by this report, the principal undertakings were: (1) to continue studies on the effect of ionic fluoride and magnesium on the role precursor phases play in apatite formation, and (2) to initiate studies on the effects of fluoride, magnesium, and carbonate on the physical texture of apatites prepared in the presence of these ions. In the former undertaking, special attention was directed to the formation of non-apatitic calcium phosphates on the surfaces of apatite crystals introduced into metastable calcium phosphate solutions and the effect fluoride and magnesium had in modifying the growth and stability of these precursor phases. In the new study, particular emphasis was given to the alterations brought about by fluoride on such physical properties of apatites as specific surface area, crystallinity, and crystal shape.

Methods Employed:

Synthetic calcium phosphate suspensions were prepared either by (1) spontaneous precipitation initiated through rapidly raising with KOH the pH of stable acidic solutions of calcium and phosphate ions to 7.4; or by (2) inoculating supersaturated solutions metastable at pH 7.4 with crystals of apatite. The suspensions were then aged at either 25° or 37°C, the pH being maintained at 7.4 with a pH-stat. Magnesium and/or fluoride ions were added either before initiating precipitation or at various stages during the aging process. Reaction kinetics were followed by monitoring solution calcium, total phosphate, hydrogen ion release, and fluoride consumption. Solid reaction products were examined chemically and by B-E-T surface area methods, x-ray diffraction and transmission electron microscopy.

Major Findings:

Two methods for preparing apatites from aqueous calcium phosphate solutions are currently in general use, namely by spontaneous precipitation from unstable supersaturated solutions or by seeding metastable solutions. Work done under this project in previous years has shown that the maturation of calcium phosphate salts spontaneously precipitated from unstable solutions at physiological pH and temperature is a complex process involving the sequential appearance of at least three distinct phases. The initial phase to separate from solution is an unstable amorphous calcium phosphate (ACP). The subsequent dissolution of this amorphous phase is accompanied by the formation of an intermediate crystalline phase similar in properties to octacalcium phosphate (OCP). This OCP phase, in turn, hydrolyzes into a stable apatitic end product. During this past year studies were carried out to determine whether this complex chain of precipitation events also occurs when exogenous apatite crystals are introduced into supersaturated but stable calcium phosphate solutions at pH 7.4. One major finding was that the course of the apatite-induced precipitation process in these metastable solutions was strongly dependent upon the degree of initial supersaturation. As an example, when at 25°C the initial calcium and phosphate concentrations equalled or exceeded 1.2 and 0.9 mM, respectively, the transient formation of an OCP-like intermediate occurred, but at initial concentrations of 0.8 and 0.6 mM or less, formation of this phase was not evident. In addition, when the degree of supersaturation favored OCP formation, fluoride and magnesium had an effect on the seeded growths comparable to that previously found in spontaneous precipitations, i.e., both ions initially retarded reaction development, which suggests that an ACP-like phase also formed on the seed surface, while the lifetime of the OCP phase which subsequently formed was extended by magnesium ions and, in contrast, shortened by fluoride. However, in seeded reactions where OCP formation did not occur, micromolar levels of fluoride accelerated reaction development from the start. No initial retardation was observed. This latter finding suggests that the increase in the rate of early growth with fluoride at low supersaturations may be related to the direct formation of fluorapatite on the seed surface. Thus the effect of small amounts of such inorganic anions as magnesium and fluoride on seeded precipitations appear to be dependent upon whether initial conditions of supersaturation were conducive for the formation of transient precursor phases such as ACP and OCP.

During this past year, studies were initiated to investigate the effects inorganic ions such as fluoride, magnesium, and carbonate have on the crystallinity of apatites grown from solution at pH 7.4. Of particular interest was the manner in which these ions can modify the size and shape of apatite crystals. The impetus behind these studies is the unusual, anisotropic manner in which fluoride is known to change the crystallinity of hard-tissue apatite. In fluoridated bone, for example, crystallinity changes are confined almost entirely to increases in particle width and thickness, with no measurable change in length. Interestingly, preliminary studies in our laboratory on synthetic systems have revealed a contrasting picture in which fluoride favors the formation of narrower, more needle-like crystals. These findings possibly reflect the importance of physiological rather than physico-chemical factors in controlling apatite growth in vivo.

Significance to Dental Research:

Hard tissue formation is a complex phenomenon involving many poorly understood processes, not the least of which is the deposition of mineral in the extracellular matrix. Because of the intimate association between mineral and matrix, it has proved difficult to study the purely physical-chemical dynamics underlying this deposition step in vivo. Consequently, to assess the importance of these physical-chemical factors it is necessary to thoroughly and systematically investigate the formation and growth of analogous calcium phosphate salts in physiological-like synthetic systems. The results obtained from such experiments during the past year indicate that the degree to which biological fluids are supersaturated with respect to calcium phosphate salts may be a critical factor in determining whether or not phases such as OCP and ACP play important intermediary roles in the mineralization of hard tissue. The fluoride-crystallinity experiments, on the other hand, serve to remind us that the dynamics of mineral formation in vivo can probably never be fully described in physicochemical terms alone, but that biological factors such as extracellular protein matrices play a major role in directing and controlling this complex process. Hopefully such knowledge may enable us to construct more appropriate model systems in the future for the study of mineral deposition in bones and teeth and of how ions such as fluoride can modify this process. Knowledge from such studies could allow us to more completely understand the origin of the anticarious properties of this anion.

Proposed Course of Project:

The current studies on the effect of inorganic anions on apatite formation, maturation, and physical properties will continue. Future emphasis will be directed toward examining how fluoride, magnesium, and carbonate, separately as well as in various combinations, can effect crystal morphology. Additional studies will be initiated to investigate the formation and stability of intermediate phases in the precipitation of calcium pyrophosphate from aqueous solutions.

2. Publications:

Meyer, J.L., Eanes, E.D.: A thermodynamic analysis of the secondary transition in the spontaneous precipitation of calcium phosphate. Calcif. Tiss. Res. 25:209-216, 1978.

Eanes, E.D., Meyer, J.L.: The influence of fluoride on apatite formation from unstable supersaturated solutions at pH 7.4. J. Dent. Res. 57:617-624, 1978.

Eanes, E.D.: Enamel apatite: chemistry, structure and properties. In, Nylen, M.U., Termine, J.D. (eds): Third International Symposium on Tooth Enamel - Growth, Structure, and Function. J. Dent. Res. 58B:829-836, 1979.

Eanes, E.D.: The influence of fluoride on the seeded growth of apatite from stable supersaturated solutions at pH 7.4. J. Dental Res. (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 DE 00162-03 LBS

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Kinetic and Thermodynamic Characterization of Calcium Phosphate Precipitation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Meyer, John L.	Research Chemist	LBS	NIDR
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	Lenk, Elaine	Visiting Associate	LBS	NIDR
	Selinger, H. Andrew	Biological Aid	LBS	NIDR
	Youmans, Patricia A.	Secretary (steno)	LBS	NIDR
	DeGraff, Barbara A.	Clerk-typing	LBS	NIDR

COOPERATING UNITS (if any)

None

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SECTION
Molecular Structure Section

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TOTAL MANYEARS: 1.90	PROFESSIONAL: 1.10	OTHER: 0.80

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this work is to determine the thermodynamic and kinetic factors which regulate the nucleation, crystal growth and maturation of calcium, phosphate crystals. This is accomplished by estimating free ionic activities in solution for all species involved in the crystallization process and relating these terms to the observed precipitation steps. A further correlation is then made between the composition of the solution and the properties of the solid calcium phosphate phase in equilibrium with it. The effect of crystallization inhibitors on the precipitation of calcium phosphates is also being studied in order to elucidate their mode of action at crystal surfaces. Emphasis is placed upon inhibitors which occur naturally in physiological systems or which are common therapeutic agents.

PHS-6040
(Rev. 10-76)

1. Project Description:

Objectives:

A number of factors appear to regulate the nucleation, crystal growth and maturation of calcium phosphate precipitates under the conditions at which biological calcification occurs. It is the purpose of this investigation to elucidate the kinetic and thermodynamic parameters which control each of the major steps involved in calcification and thereby gain additional insight into the mineralization process itself. Emphasis is placed upon the solution phase and how its composition affects crystallization processes, although the isolated solid phases are also studied with conventional chemical, microscopic and spectroscopic techniques.

Methods Employed:

The calcium phosphate precipitations are performed under conditions of constant temperature and pH. From the analytical concentrations of the reactants, free ionic concentrations and chemical activities of each ionic species are calculated using known thermodynamic equilibrium constants and computed activity coefficients. A computer program has been developed to perform these calculations. Knowledge of the free ionic activities of each species involved in the precipitation provides the necessary kinetic and thermodynamic information for the correlation between events that occur in solution versus those that occur in the solid state. The solid material is isolated from the well-characterized solutions by Millipore filtration and lyophilized. The calcium phosphate precipitates are analyzed for other possible lattice constituents (i.e. acid phosphate, carbonate, and hydroxide) as well as the calcium and phosphate contents.

Major Findings:

The spontaneous precipitation of calcium phosphate is characterized by the formation of an initial phase which is amorphous with respect to x-ray diffraction (amorphous calcium phosphate, ACP). If left in contact with solution, ACP transforms into a crystalline material with an apatitic-like x-ray diffraction pattern, but which has recently been shown to have the thermodynamic properties of octacalcium phosphate (OCP). Further experiments have shown that the OCP-like crystalline phase undergoes a secondary transformation to a more basic crystalline material with a tricalcium phosphate (TCP) stoichiometry. This latter phase gradually matures to hydroxyapatite (HAP) the stable form of calcium phosphate under physiological condition. Since the previous results suggested that the first formed crystalline phases were deficient in hydroxide ion (i.e. approximating the composition of either OCP, $Ca_4H(PO_4)_3$, or TCP, $Ca_3(PO_4)_2$, compared to that expected for HAP, $Ca_5(PO_4)_3$ OH. a titration method was developed to directly analyze the precipitates for their hydroxide content. Once corrections were made for the presence of the other titratable materials in the solid phases (phosphate, acid phosphate, and carbonate) it was found that the early crystalline calcium phosphate phases generally contain from 0 to 20 percent of the theoretical amount of hydroxide for HAP, which is consistent with the results of the thermodynamic studies. Surprisingly, it was found that even the mature apatitic materials also contained less hydroxide than expected for pure HAP. In fact, few crystalline calcium phosphates were isolated which contained more than 50 percent of the theoretical amount. The significance of this latter observation is not clear at present but the suggestion is made that hydroxyl is not necessary for the integrity of the HAP structure. In fact, the most stable apatitic material formed under simulated biological conditions would appear to be one with at least half the hydroxyl positions either vacant or filled with another species, possibly water.

Significance to Dental Research:

A knowledge of the factors that influence calcium phosphate precipitation is required for a complete understanding of the physiological processes that result in hard tissue mineralization. A thermodynamic approach to the study of calcium phosphate precipitation under simulated *in vivo* conditions yields basic information that can be related ultimately to conditions that may exist in vital fluids in contact with the mineral phase. The combination of thermodynamic and kinetic methods can provide a better description of those dynamic processes resulting in physiological and pathological calcifications or decalcifications within the body.

Proposed Course of Project:

Experiments designed to characterize the entire course of the precipitation of calcium phosphate from the initial formation of ACP to its eventual transformation to crystalline hydroxyapatite will continue. Solutions in contact with the solid phase will be analyzed by chemical thermodynamic methods to obtain information on the nature of the various calcium phosphate phases formed. All crystalline materials will be analyzed by conventional techniques to assure that the thermodynamic analysis results are consistent with the properties of the solid phase. Crystal growth inhibitors are known to affect crystallization of calcium phosphates but a detailed analysis of their effects on all the various phase transitions has not yet been made. Because of the potential biological significance of mineralization inhibitors on intra- and extracellular calcification, future work will concentrate on these substances with particular emphasis on their mechanism of action at crystal surfaces.

The relationship between the formation of the organic and mineral phases in hard tissues will be investigated with particular emphasis placed on determining how the presence of one phase affects the initial separation (nucleation) of the other phase from an unstable supersaturated solution. Transmission electron microscopy will be used to study the morphological relationship between the organic and inorganic phases.

2. Publications:

Meyer, J.L. and Eanes, E.D.: A thermodynamic analysis of the secondary transition in the spontaneous precipitation of calcium phosphate. Calcif. Tiss. Res., 25: 209-216, 1978.

Eanes, E.D. and Meyer, J.L.: The influence of fluoride on apatite formation from unstable supersaturated solutions at pH 7.4. J. Dent. Res., 57; 617-624, 1978.

Meyer, J.L.: Hydroxyl content of solution-precipitated calcium phosphate. Calcif. Tiss. Intl., 27, 153-160, 1979.

Meyer, J.L. and Selinger, A.H.: The effect of citrate on calcium phosphate phase transitions. Mineral Electrolyte Metab., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 DE 00204-03 LBS

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Collagenous Matrix and Bone Differentiation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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	Martire, Diane	Biological Aid	LBS	NIDR
	Brown, Josephine	Student-trainee, Bio. Aid	LBS	NIDR
	Smith, Edith	Student-trainee, Bio. Aid	LBS	NIDR
	Woodson, Kevin	Student-trainee, Bio. Aid	LBS	NIDR
	Youmans, Patricia A.	Secretary-steno	LBS	NIDR
	DeGraff, Barbara A.	Clerk-typing	LBS	NIDR
	Floyd, Steven W.	Photographer	LBS	NIDR

COOPERATING UNITS (if any)

LDBA, NIDR and Metabolism Branch, National Cancer Institute, Bethesda Maryland 20205

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Laboratory of Biological Structure

SECTION
Molecular Structure Section

INSTITUTE AND LOCATION
NIDR, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 5.36	PROFESSIONAL: 2.75	OTHER: 2.61

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The objective of this project is to investigate extracellular matrix-cell interactions employing an experimental system of matrix-induced endochondral bone differentiation. This experimental model further affords a method to undertake systematic studies on the biochemistry and physiology of endochondral bone formation. Subjects currently under investigation are: (1) the local mitogenic effects of collagenous bone matrix; (2) changes in proline-synthetic and degradative enzymes during matrix-induced cartilage and bone development; (3) immunofluorescent localization of type IV collagen and laminin during endochondral bone differentiation and regulation by pituitary growth hormone; (4) influence of magnesium depletion on endochondral bone formation (5) influence of experimental diabetes and insulin on cartilage and bone differentiation; and (6) role of collagen-crosslinking in mineralization of bone.

PHS-6040
(Rev. 10-76)

1. Project Description

Introduction and Background:

The origin and evolution of multicellular organisms were marked by the appearance and specialization of extracellular matrices. The extracellular matrix is predominantly composed of collagens, proteoglycans and glycoproteins. Of all the tissues in the body only bone, cartilage, tendon and tooth exhibit vast expanses of extracellular matrix. While we know that all extracellular matrices are products of cellular biosynthetic activity, we know very little about the interactions and feedback between matrix and cells. Our studies have concentrated on the area of collagenous bone matrix-cell interactions.

We have developed a useful experimental method to investigate several aspects of endochondral bone differentiation. Subcutaneous transplantation of demineralized rat bone or tooth matrix results in induction of bone formation locally. The sequential cellular changes are: (1) transient chemotaxis for leukocytes (days 1-2); (2) a more prolonged chemotaxis for fibroblasts (days 2-3); (3) cell proliferation (days 3-5); (4) chondrogenesis (days 5-7); (5) hypertrophy and calcification of cartilage (days 9-10); (6) osteogenesis and bone mineralization (days 11-14); (7) remodeling of the ossicle (days 14-18); (8) differentiation of hematopoietic bone marrow (days 18-24).

The induced bone and bone marrow persist indefinitely in a functional state. The temporal sequence is highly reproducible and we have extensively studied the factors influencing the sequence. This experimental model is the mainstay of our work and affords a system to dissect the main events in endochondral bone formation.

Experimental Methods:

Diaphyseal shafts are prepared from adult rats by standard techniques. Pulverized matrix particles of uniform size are then demineralized prior to implantation. The usual repertoire of standard biochemical laboratory techniques such as density gradient centrifugation, column chromatography and radioisotopic tracer methodology are extensively employed.

The Local Mitogenic Effects of Collagenous Bone Matrix:

In our continuing endeavor to elucidate the mechanism of action of bone matrix on responding mesenchymal cells we have discovered the local mitogenic effects of the collagenous matrix. The importance of collagens in growth and differentiation and in cell adhesion is well documented. One of the early responses to implanted collagenous matrix is the appearance of connective tissue mesenchymal cells adjacent to the implanted matrix before their differentiation into cartilage. We found that on days 3 and 8-9 after implantation there is an increase in the activity of ornithine decarboxylase (ODC), an enzyme involved in polyamine biosynthesis and considered a marker for cell proliferation. These findings have been corroborated by incorporation of ^{3}H-thymidine into acid-precipitable fractions. Autoradiography at the light microscope level revealed mesenchymal cells labelled with ^{3}H-thymidine in the vicinity of the implanted matrix. These results imply a role for collagenous matrix in anchorage-dependent events in cell growth and differentiation. Additionally, these experiments may explain the local nature of fracture and wound healing responses and also provide a basis for further studies in clinical areas such as dental implants and periodontal diseases.

Changes in Proline Synthetic and Degradative Enzymes During Cartilage and Bone Formation:

Proline, a characteristic constituent of collagens, can be synthesized from either glutamic acid or ornithine through the formation of intermediate pyrroline-5-carboxylate. Proline may be degraded to glutamic acid. Thus mammalian tissues have the potential for net biosynthesis of proline and for generation from proline of urea-cycle and tricarboxylic acid cycle intermediates. In order to characterize the tissue-specific patterns of proline biosynthesis and degradation in collagenous tissues such as cartilage and bone, we have examined the proline biosynthetic and degradative enzymes in matrix-induced cartilage and bone development systems. Ornithine aminotransferase and pyrroline-5-carboxylate reductase, the biosynthetic enzymes, have peaks of activity that correlate with maximal synthesis of type II collagen on day 7 by chondrocytes. Both enzymes also are active during bone formation. In contrast proline oxidase and pyrroline-5-carboxylate dehydrogenase, the degradative enzymes, are present at very low levels that do not change as new cell types appear. These findings substantiate the importance of proline biosynthesis in collagenous tissue development. These investigations were done in collaboration with Drs. R.J. Smith and J. M. Phang of the Metabolism Branch, NCI, NIH.

Localization of Type IV Collagen and Laminin During Bone Differentiation and Regulation by Pituitary Growth Hormone:

The pituitary growth hormone is essential for normal bone development and mineralization. However, the mechanisms underlying this action and its target cells are not well defined. The formation of endochondral bone requires a progression of cell types. Vascularization, though little understood, is an important prerequisite for osteogenesis.

Current evidence indicates that vascular invasion provides the two major bone stem cell populations: osteoclasts, derived from blood-borne hematogenous elements (monocytes), and osteoblasts, probably derived from vascular endothelial cells or perivascular connective tissue cells. We have monitored vascularization during matrix-induced osteogenesis by the immunofluorescent localization of type IV collagen and laminin, a noncollagenous glycoprotein isolated from basement membrane producing tumors. These experiments were performed in collaboration with Dr. J.-M. Foidart, LDBA, NIDR. These two antigens were localized in the vascular basement membrane of proliferating capillaries invading the osteogenic plaques. A close correlation was found between capillary invasion and subsequent bone formation as assessed by alkaline phosphatase activity and ^{45}Ca incorporation into bone mineral. The vascular invasion was reduced and delayed in hypophysectomized recipients. However, this deficiency could be ameliorated by administration of pituitary growth hormone. These results suggest that growth hormone could modulate osteogenesis by regulating vascular invasion.

Influence of Magnesium Depletion on Bone Formation and Mineralization:

The effect of magnesium deficiency on bone cell differentiation and bone formation was investigated using *in vivo* matrix-induced endochondral ossification as a test system. Demineralized bone matrix was implanted subcutaneously in young (35 day old) male Long-Evans rats that had been fed a semi-synthetic Mg deficient diet (50 ppm Mg) for 7 days. Plasma Mg levels were reduced to 25-30 percent of control values at that time. Control rats were pair fed the same diet, supplemented to contain 1000 ppm Mg. The implants were harvested 7, 9, 11, 15 and 20 days after implantation and analyzed for Mg and Ca content, ^{45}Ca incorporation and alkaline phosphatase levels. At each stage, plaque (implants) removed from Mg deficient rats showed retardation in cartilage and bone differentiation and matrix calcification. Magnesium content was markedly reduced when compared to the control plaques. Histological appearance of the matrix-induced plaques confirmed the retardation in bone development and mineralization suggested by the chemical indicators. Most marked was the virtual absence of bone marrow in 20 day old plaques in Mg depleted rats. These data show that bone cell differentiation can occur in a severely Mg depleted environment, although the onset of mineralization and bone remodeling was delayed and bone marrow differentiation was impaired.

Influence of Experimental Diabetes and Insulin on Cartilage and Bone Differentiation:

It is well known that the diabetic state results in complications of periodontal tissues and in generalized osteoporosis. However, the precise role of insulin in differentiation and metabolism of cartilage and bone is not known. In order to gain further insights into this problem we have studied the influence of diabetes and insulin on endochondral bone development. The effect of streptozotocin-induced diabetes on discrete stages of matrix-induced endochondral bone formation has been investigated. Demineralized bone matrix was implanted subcutaneously to induce local endochondral bone development. Three days following matrix implantation, mesenchymal cell proliferation was inhibited in diabetic rats as evidenced by a 65% reduction of ornithine decarboxylase (ODC) activity and a 56% reduction of [^{3}H]-thymidine incorporation per µg DNA compared to non-diabetic controls; the inhibition was prevented by insulin treatment. In diabetic animals chondrogenesis on day 7 was reduced by 49% compared to control animals as assessed by $^{35}SO_4$ incorporation into proteoglycans. Exogenous insulin was stimulatory to cartilage development when present during days 0 through 4 (mesenchymal cell proliferation). Calcification of cartilage and osteogenesis were monitored by alkaline phosphatase activity and ^{45}Ca incorporation. Alkaline phosphatase attained a peak value in control rats on day 11. In diabetic rats the peak was on day 14. In the diabetic group ^{45}Ca incorporation was less than 50% of control throughout the duration of the experiment. The reduction in alkaline phosphatase activity and ^{45}Ca incorporation were prevented by insulin. Decreased in vivo endochondral bone growth and development during diabetes is the result of (1) inhibition of insulin-dependent mesenchymal cell proliferation (2) decreased and delayed cartilage formation due to impaired mesenchymal cell proliferation (3) decreased and delayed vascular invasion prior to chondrolysis and osteogenesis and (4) reduced insulin-dependent calcification and ossification.

Inhibition of Mineralization by Experimental Lathyrism During Endochondral Bone Differentiation:

The various types of collagens have been implicated in cell attachment, cell differentiation and morphogenesis. Cross-linked collagenous matrix appears to be a prerequisite for optimal mineralization. The availability of β-aminopropionitrile (BAPN) and other agents that inhibit collagen cross-linking enables an experimental scrutiny of the influence of lathyrism on bone differentiation and mineralization. The influence of BAPN on mineralization during endochondral bone development was investigated. Mesenchymal cell proliferation was not affected by the lathyritic agent. However, chondrogenic differentiation on day 7 was inhibited by about 45-50% by BAPN as indicated by $^{35}SO_4$ incorporation into proteoglycans. On days 9 and 14 cartilage and bone mineralization, respectively, as indicated by ^{45}Ca incorporation, were profoundly inhibited by BAPN treatment.

The initial rate and the final extent of mineralization were impaired by lathyrism. These results demonstrate the critical role of adequately cross-linked collagenous matrix for mineralization of cartilage and bone.

Significance to Dental and Medical Research:

A detailed knowledge of bone induction by cell-free collagenous bone matrix has immense implications for fracture healing, and other orthopedic diseases and in the realm of oral implants. In cancer, impaired matrix-cell interactions lead to metastases. Our experimental model represents a prototype for studying matrix-cell interactions and may shed light on the mechanisms involved in normal physiology and in pathogenesis.

2. Publications:

Faltz, L.L., Reddi, A.H., Hascall, G.K., Martin, D., Pita, J., and Hascall, V.C.: Characteristics of proteoglycans extracted from the Swarm rat chondrosarcoma with associative solvents. J. Biol. Chem. 254:1375-1380, 1979.

Rath, N.C. and Reddi, A.H.: Collagenous bone matrix is a local mitogen. Nature 278:855-857, 1979.

Rath, N.C. and Reddi, A.H.: Influence of adrenalectomy and dexamethasone on matrix-induced endochondral bone differentiation. Endocrinology 104:1698-1704, 1979.

Smith, R.J., Reddi, A.H. and Phang, J.M.: Changes in proline-synthetic and degradative enzymes during matrix-induced cartilage and bone formation. Calcified Tissue International 27:275-279, 1979.

Foidart, J.M. and Reddi, A.H. Immunofluorescent localization of type IV collagen and laminin during endochondral bone differentiation and regulation by pituitary growth hormone. Develop. Biol. (in press).

Schwartz, R. and Reddi, A.H.: Influence of magnesium depletion on matrix-induced endochondral bone formation. Calcified Tissue International (in press).

Reddi, A.H. and Sullivan, N.E. Inhibition of mineralization by experimental lathyrism during matrix-induced endochondral bone differentiation. Proc. Soc. Exp. Biol. & Med. (in press).

Rath, N.C. and Reddi, A.H.: Evaluation of anti-inflammatory drugs based upon the inhibition of matrix-induced ornithine decarboxylase activity during connective tissue proliferation. Proc. Soc. Exp. Biol. & Med. (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 DE 00251-02 LBS

PERIOD COVERED October 1, 1978 to September 30, 1979 Y01-DE-80027

TITLE OF PROJECT (80 characters or less)

Microprobe Analysis of Developing Rat Enamel

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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LAB/BRANCH Laboratory of Biological Structure

SECTION Molecular Structure Section

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.25	1.05	0.2

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The objectives of this study are the identification and characterization of the inorganic phosphate and carbonate phases and the organic matrix of rodent incisor enamel, and changes in these components which occur during mineralization and maturation. Inorganic and organic phosphate and carbonate phases localized in the tissue during development are analysed using spectroscopic microprobe techniques. The Raman microprobe, capable of identifying molecular species, and elemental microprobe analyses (x-ray microanalysis and ion microprobe analysis) used in conjunction allow the correlation of data obtained from these two types of complementary analyses. This data then, may be interpreted to discern the distribution patterns of calcium, phosphate, magnesium carbonate and other ions at mineralizing sites in developing enamel.

1. Project Description

Objectives:

The objectives of this study include the following: (1) to discern the accumulation and describe the distribution of inorganic (calcium, phosphate, magnesium and carbonate) and organic (especially organically bound phosphate) components during the mineralization and maturation of hard tissue; (2) to correlate the accumulation of the inorganic phase of hard tissue with the changes that occur in the organic matrix, i.e. its concentration and secondary structure; and (3) to associate this mineralization pattern as described by (1) and (2) above with the varying stages of cellular function.

Methods:

The techniques used in this study are microprobe methods - the Raman electron and ion microprobes. Microprobe methods are especially suited to the analysis of these spatially heterogeneous biological samples. The advantages of these methods are: (1) through the interpretation of Raman spectra and elemental analyses, various molecular species may be identified and correlated with an elemental analysis; and (2) the resolution of the methods on fresh frozen tissue sections is approximately 5 µm and therefore allows an analysis of approximately 10^{-9} gms of material. As applied to our samples, discrete analyses are performed on previously unattainable samples without the disadvantage of sample isolation and pooling, and therefore the results of this technique may be correlated directly with the histological structure.

Samples used in these studies:

Two aspects of biological mineralization which are of interest to this study are the characterization of the matrix and the initial crystallite at the site of mineralization and the changes which occur in these phases as the tissue matures. Therefore, samples which contain these areas of interest as discrete, easily identifiable regions are suitable to the study. These include the following: (1) the continuously erupting rat incisor in which both amelogenesis and dentinogenesis may be studied; (2) the epiphyses in embryonic tibia as a system for both chondrogenesis and osteogenesis; and (3) matrix-induced bone plaques, which allow the sequential study of discrete stages of endochondral bone formation such as deposition and calcification of the cartilage matrix during chondrogenesis, dissolution of the cartilage during chondrolysis, bone matrix formation and mineralization, and have remodeling. Further experimental details of this system may be found in the report of A.H. Reddi (Z01 DE-00204-03).

Sample Preparation:

All samples consist of thin sections cut from frozen unfixed tissue as described in last years report. The protocol, which allows precise analysis of microregions, is the following: (1) 5-30 μm frozen sections cut on a cryostat are transferred to the appropriate Raman microprobe substrate (either lithium fluoride or sapphire) and air dried. Alternate serial sections are stained employing conventional histological procedures; (2) photomicrographs of the sections are recorded; (3) Raman microprobe data are collected on specific areas (normally 5 μm in diameter) of the section; (4) the same section may then be carbon coated for x-ray microanalysis and/or ion probe analysis of the same regions; and (5) these sections may be further stained for histological study.

Raman analysis of a region in these samples results in a spectrum characteristic of the components present. From a priori knowledge of the major components, the total spectrum must be resolved or decomposed into the spectra of the various components. This has been accomplished by observing the spectra of both pure materials and those separated from tissue through biochemical techniques. Once the spectral features of these components have been assigned or determined, changes in concentration may be followed by comparison of the results from one region to another.

Major Findings:

Inorganic Components:

1. In rat incisor enamel, dentin and embryonic bone, in regions of very low mineralization, the earliest phosphate mineral phase detected has a ν_1 P-O stretching mode at 960 cm^{-1}. This is in good agreement with the ν_1 for an orthophosphate ion in the apatite lattice. Comparison of the Raman spectrum of these regions with the Raman spectrum of well characterized phosphates such as octacalcium phosphate or dicalcium phosphate reveals no evidence for their presence as precursor phases to biological apatite.

2. In mineralizing enamel, within a few micrometers from the ameloblast, portions of the Raman spectrum of the mineral huntite, $Mg_3Ca(CO_3)_4$, have been consistently observed. In these regions the phosphate intensity is either very weak or nonexistent. $Mg_3Ca(CO_3)_4$ is a mineral with a unit cell very similar to that of apatite, with carbonate ions in nearly equivalent positions of the phosphate and hydroxyl ions in the apatite lattice. This similarity in the crystal structures would permit, as our preliminary in vitro studies suggest, the epitaxial growth of phosphate mineral on a core of carbonate. The association of this carbonate phase with magnesium and the resulting

morphology correlates well with the preferential removal of both magnesium and carbonate during carious attack in vivo, and the preferential dissolution of the central core of enamel crystallites in vitro. These results also suggest that the initial mineral phase is a carbonate and may be closely linked to the metabolism of the cells. As the enamel becomes increasingly mineralized phosphate accumulates as biological apatite.

3. In embryonic bone and mineralizing dentin, similar observations have been made concerning both the phosphate and carbonate phases.

4. Electron microprobe analyses of these identical areas have revealed low calcium and phosphorus content. However, we have not been able to observe magnesium reproducibly. Based on our calculations, the magnesium concentration (considering the magnesium is present as huntite) is at the limit of sensitivity of the method. Present efforts are being directed toward improving our technique of sample preparation for the ion microprobe, which has a greater sensitivity for magnesium.

Organic Matrix:

1. The most striking difference in the Raman spectra of collagenous (bone and dentin) and noncollagenous mineralizing tissue, is the Amide III region of the spectrum. This region is diagnostic of protein secondary structure. In enamel matrix, the spectra indicate that the majority of the protein has an antiparallel β-pleated sheet configuration. In bone and dentin, the spectra are typical of the triple helix of collagen.

2. The portion of the Raman spectrum associated with the carboxyl stretching modes and CH_2 wagging modes of glutamic and aspartic acids, two amino acids indicative of early mineralized matrix, have been identified. Thus, the presence of the protein associated with high glutamic acid content may be followed and associated with the inorganic phase concentration.

3. Bands have been identified that are associated with the ring stretching mode of either adenosine or guanosine. In the last report it was suggested that a portion of the phosphate spectrum was due to a covalently bound orthophosphate ion, probably a phosphoprotein. With the identification of a nucleoside, either adenosine or guanosine, it is likely that a major portion of the organic phosphate is a nucleotide. This suggests the presence of an associated enzyme system for inorganic phosphate, which may regulate the growth of the apatite crystallites.

Proposed Course:

1. Samples of the mineral huntite will be prepared. These will be used as seeds upon which calcium phosphate will be allowed to grow from metastable solutions. This material will be analyzed spectroscopically to determine if the phosphate changes the structure of the underlying huntite via a solid state reaction. If this were to occur, i.e. sufficient carbonate and phosphate were to interdiffuse to eliminate the structural attributes of huntite while maintaining a high concentration of carbonate in the original seed material, it would explain the loss of the huntite spectra and account for the carbonate spectrum of mature hard tissue.

2. Based on the relative intensity of the Raman bands associated with (a) the antiparallel β-pleated sheet, (b) the nucleoside, (c) the organic phosphate, (d) the inorganic phosphate, and (e) the carbonate component, their concentrations will be mapped out histologically in the four mineralizing tissues described above.

3. From preliminary experiments on mineralizing cartilage, it has been determined that the sulfate component of the proteoglycans of cartilage has a very distinctive spectrum. These studies will be continued, especially in the matrix-induced plaques of Dr. A.H. Reddi and growth plate cartilage.

4. Ion probe analyses are to be continued to determine the $Mg:CO_3$ ratios in mineralizing tissues.

2. Publications:

Casciani, F.S., Etz, E.S., Newbury, D.E. and Doty, S.B.: Raman Microprobe Studies of Two Mineralizing Tissues: Enamel of the Rat Incisor and the Embryonic Chick Tibia. Scanning-Electron Microscopy/ 1979/II 383-391.

Casciani, F.S. and Etz, E.S.: Raman Microprobe Study of Biological Mineralization in Situ: Enamel of the Rat Incisor. Proceedings of Microbeam Analysis Society (in press).

Summary Statement
Laboratory of Microbiology and Immunology
National Institute of Dental Research

The major emphasis in our research programs has remained virtually unchanged since FY 1978. Our activities include the following areas of investigation: 1) The role of soluble mediators derived from lymphoreticular cells in the induction of immunity and inflammation, 2) the regulation of connective tissue metabolism by cellular and humoral immune mechanisms, 3) clinical immunological studies on autoimmune and periodontal diseases, 4) genetic determinants in the host response to bacterial endotoxin, 5) the molecular biology, physiology and taxonomy of oral bacteria and 6) mechanisms of microbial pathogenicity ultimately related to periodontal diseases and dental caries. A more detailed description of the specific research related to these areas can be found in the individual reports. The following is a resumé of our progress during the past year.

Although the microbial composition of dental plaque is complex, current research in our *Microbiology Section* has established that highly specific interactions occur among certain of the resident microflora. *Streptococcus sanguis* is one of the first species of bacteria to colonize the enamel portion of the tooth surface. Last year we reported that a number of laboratory strains of *Actinomyces viscosus* and *Actinomyces naeslundii* can attach to *S. sanguis* while strains of *Actinomyces israelii* did not. In expanding this study to include over 100 human isolates of actinomycetes, a total of six biochemically distinguishable patterns of coaggregation have been identified.

A. israelii, an inhabitant of the more anaerobic environment of periodontal plaque, does, however, interact with certain gram negative gliding bacteria (*Cytophaga* sp.) that are also found in such plaque. The interactions observed between these two groups of organisms are specific too, and *Cytophaga* sp. do not coaggregate with *A. viscosus* or *A. naeslundii*. The *Cytophaga* sp. isolated from periodontal plaque also exhibit a unique and specific affinity for colonizing the root surfaces of teeth. *In vitro* assays revealed that demineralization of the tooth surface resulted from such colonization. Recent studies have shown that the initial attachment of the organisms to hydroxyl apatite surfaces is mediated by certain lipoprotein components contained within the outer membrane of the bacterial cell.

Other efforts are being directed towards defining the enzymatic mechanisms responsible for the regulation of sugar metabolism and glucan biosynthesis in oral streptococci. Concerted studies have shown that sucrose is metabolized by an inducible sucrose-dependent phosphoenolpyruvate phosphotransferase system which transports and simultaneously phosphorylates the glucose moiety of sucrose. The resultant sucrose 6-phosphate is then cleaved to fructose and glucose 6-phosphate by a constitutively synthesized sucrose 6-phosphate hydrolase. Biochemical studies indicate that the sucrose 6-phosphate hydrolase is probably responsible for the intracellular invertase activity reported by other workers in *Streptococcus mutans* and other oral streptococci.

Using Streptococcus salivarius as a model system, regulation of the biosynthesis and secretion of glucosyltransferase is being studied. Production of the extracellular glucosyltransferase complex is completely inhibited by cerulenin, an inhibitor of fatty acid biosynthesis. This antibiotic has no effect on DNA, RNA or protein synthesis, but does inhibit cellular incorporation of acetate. The secretion of glucosyltransferase appears to be closely related to long chain fatty acid synthesis.

By encoding the information necessary for the transcription of enzyme biosynthesis on plasmids, bacteria gain the advantage of being able to produce significantly higher levels of these protein catalysts than microbes that carry such information chromosomally. The genetic disposition of information that codes for the production of enzymes catalyzing the metabolism of two important dietary sugars, sucrose and lactose, are currently being investigated. In certain streptococci and lactobacilli one or more of the enzymes responsible for the dissimilation of lactose appears to be plasmid borne. Preliminary evidence suggests that sucrose metabolism may also be plasmid mediated in these same organisms. Plasmids appear to play a major role in the metabolism of other carbohydrates in the Lancefield group N streptococci and probably play a minor role in the transmission of tetracycline resistance in oral streptococci.

Three distinct approaches are being used by the Cellular Immunology Section to investigate the basic host defense mechanisms that are involved in mounting inflammatory reactions in diseases such as periodontal disease and rheumatoid arthritis.

Lymphocytes, monocytes and cell lines secrete a number of mediators which modulate cellular participation in inflammation. These mediators are recovered from the supernatants of cultured cells. Isolation and characterization of these factors should increase our understanding and consequent capacity to manipulate inflammatory reactions. The factors that are being investigated include lymphokines and cytokines such as lymphocyte derived mitogenic factors (LMF), osteoclast activation factor (OAF), monocyte derived lymphocyte activating factor (LAF) and tumor necrosis factor (TNF), as well as fibroblast cell line derived colony stimulating factor(s) (CSF) and interferon (IF). These factors are hormone-like glycopeptides ranging in MW from 10,000-200,000 and are biologically very active at low (ng) concentrations. A major effort is underway to use cell lines to produce some of these factors in quantities which are sufficient for isolation, characterization and to obtain antibodies to them. LAF obtained from a mouse cell line has been purified by a factor of 10,000-20,000.

Considerable progress is being made in defining the biological role in host defense of some of these mediators. A number of these molecules amplify the afferent limb of the inflammatory response. For example, LAF has been found to stimulate the proliferation as well as functional maturation of thymocytes and lymphocytes. It promotes antibody production and enhances the development of cytotoxic lymphocytes. In addition, LAF stimulates nonlymphoid fibroblast-like cells which are present in synovial tissue of inflamed joints to produce prostaglandins and collagenase. Thus LAF is involved in both immunological and non-immunological pathways that augment inflammatory reactions. LMF is a lymphokine which has multiple and diverse biological effects on lymphocytes. It promotes lymphocyte proliferation, the development of cytotoxic lymphocytes, supports the growth of normal lymphocyte lines and enhances antibody production. Our investigations also indicate that colony stimulating factors not only promotes the proliferation of precursors of granulocytes and monocytes, but also have a maturational effect on the progeny so that they can function in inflammatory reactions by elaborating products such as LAF and prostaglandins.

Some of the mediators that are being studied participate in the efferent aspects of inflammation and have a direct effect on nonlymphoid target cells. This includes macrophage-derived TNF which is selectively cytotoxic for tumor cells in vivo and in vitro. Lymphocytes can be activated to produce OAF which stimulates bone resorption. This factor is of particular interest since it is implicated in the bone resoprtion seen in periodontal disease. It is being studied intensively in conjunction with investigators at the Walter Reed Army Institute of Dental Research who are funded by an Interagency Agreement Contract (Y01-DE-60025) from NIDR. Our studies of lymphokines and related factors is gaining increasing scientific recognition. LMI investigators participated in the organization, chairing and scientific interchange at the recently held 2nd International Workshop of Lymphokines. They have also coedited and contributed several chapters to a recently published reference text entitled "Biology of the Lymphokines".

A second program in the section centers on studies of the role of oxygen intermediates in inflammation. Both dying cells and cells actively engaged in inflammatory reactions produce cytotoxic superoxide anions, hydroxyl radicals and hydrogen peroxide. Using in vitro tissue culture models, we have found that the immunosuppressive effect of activated macrophages is due to excessive production of both prostaglandins and hydrogen peroxide. The deleterious in vitro effects of these oxygen intermediates is counteracted by "scavenger" molecules such as catalase, reduced glutathione and 2-mercaptoethanol. Many more "scavengers" are currently being tested, with a view to finding some with therapeutic utility in reducing some of the self-destructive consequences of severe inflammation.

The third major activity of the section continues studies of the multitudinous immunological effects of the lipopolysaccharide endotoxin (LPS) moiety of gram negative bacteria. Adoptive transfer of bone marrow cells from LPS sensitive mice (C3H/HeN) to the mutant (autosomal codominant) LPS unresponsive C3H/HeJ mice was able to restore their LPS reactivity. The reconstituted C3H/HeJ mice became susceptible to the adjuvant and lethal effects of LPS. In vitro studies showed that macrophages, lymphocytes and fibroblasts from C3H/HeJ all failed to be activated by LPS. By contrast, normal mouse cells when stimulated by LPS produced a variety of potent factors that presumably are responsible for the phenomenon of endotoxicity. These factors include prostaglandins, LAF, IF, CSF and serum amyloid associated protein, an acute phase reactant. Normal macrophages also can be activated by LPS to kill tumor cells and to secrete tumor necrosis factor (TNF) which is toxic for tumor cells. Thus LPS is an important inducer of inflammatory reactions and studies of its effects elucidate basic mechanisms involved in host reactions to infectious organisms.

The Humoral Immunity Section has extended its investigations concerning immunological mechanisms in the regulation of connective tissue metabolism. Synthesis of collagen, a major constituent of connective tissue, may be, in part, under immunological control. A factor(s) present in the supernatants of stimulated lymphocytes and macrophages enhances the synthesis of collagen as well as non-collagen protein by fibroblasts which subsequently undergo cell division. Delineation of the early cellular events leading to the production of these proteins is receiving major emphasis. cAMP and prostaglandin are implicated in this activation sequence since within a few hours after exposure to active lymphocyte or macrophage supernatants, both intracellular cAMP and media prostaglandin levels are elevated in cultures of fibroblasts. Characterization of this "fibroblast activating factor" and its relationship to other biologically active molecules derived from lymphocytes and macrophages are presently being investigated.

Immunological responses may also contribute to the catabolism of connective tissue. The previously described dependence on prostaglandin synthesis for the production of collagenase by activated macrophages has now been shown to be related to an elevation of cAMP in these cells. This requirement for prostaglandin synthesis can be bypassed by the exogenous addition of the cyclic nucleotide analog, dibutyryl cAMP. In addition, agents such as cholera toxin which elevate cAMP at pharmacological concentrations enhance the production of collagenase by activated macrophages.

The humoral immune system also contributes to the destruction of connective tissue. Evidence has now been obtained which suggests that complement activation by immunoglobulins reactive with cell surface antigens present in bone has biological consequences in addition to the initiation of prostaglandin synthesis with resultant destruction of the bone. This effector system also inhibits the growth of bone as determined by decreased synthesis of both collagen and non-collagen protein. Of interest is the finding that although the inhibition of bone growth by complement sufficient serum is accompanied by enhanced prostaglandin synthesis, these two events are apparently unrelated since agents which block the production of the prostaglandins do not restore the growth of bone.

Studies in collaboration with the Microbiology Section have concentrated on delineation of the mechanisms by which microorganisms adhere to other microorganisms and mammalian cells. Interactions between strains of oral actinomycetes and streptococci occur via lectin-like sites on one species which react with carbohydrate receptors on the other species.

Current work in this Section is focusing on the immunochemical characterization of the lectin involved in these reactions and has identified the carbohydrate specificities to be lactose and B-methyl galactoside. In collaboration with our Clinical Immunology Section monoclonal antibodies have been produced against the bacterial structures which carry the lectin binding sites thus providing reagents which will definitively characterize the cell wall components involved in adherence. In addition, these latter studies may be extremely helpful in defining more precisely the role of antibodies in host resistance through their interactions with coaggregates of dental plaque bacteria.

The Clinical Immunology Section continues to investigate genetic and immunological factors in the pathophysiology of Sjögren's Syndrome. During the past year the current knowledge about this disease was reviewed at one of the Combined Clinical Conferences and will be published in the Annals of Internal Medicine. Clinical studies demonstrated that Sjögren's Syndrome either alone or associated with systemic lupus erythematosus are closely related, but differ from Sjögren's Syndrome associated with rheumatoid arthritis. These diseases also have differences in the distribution and specificity of the autoantibodies present in the patient's serum. Patients with Sjögren's Syndrome have auto-antibodies directed to SS-A and SS-B antigens, but not to RANA; in contrast patients with rheumatoid arthritis associated with Sjögren's Syndrome produce antibodies to RANA but not SS-A or SS-B antigens. These two groups of patients have common genetic markers (B-lymphocyte Ia-markers Ia-172 and Ia-350), but differ in some other immunogenetic markers (HLA-B8, HLA-DRW3, HLA-DRW4 and Ia-715). The results suggest that the lymphocytic infiltration of the salivary glands may depend upon the Ia-immune response genes.

Immunological studies are also attempting to better define the pathological mechanisms in Sjögren's Syndrome. The sera of these patients have immune complexes and there is defective in vivo clearance of immune complexes. The patients with the most severe defective clearance have the most prominent disease. Studies with lymphocyte subpopulations demonstrate normal total lymphocytes, T and B cell numbers although there is a decreased relative proportion of T_G cells among patients with extraglandular disease. This was found to be due to a serum factor which reversibly blocked the expression of the IgG Fc receptor of the T_G cells. However, unlike the suppressor cell defect in patients with systemic lupus erythematosus, there are no abnormalities in mitogen generated suppression. This difference in immunoregulation between systemic lupus erythematosus and Sjögren's Syndrome may explain the differences in the severity and expression of these autoimmune reactions.

Studies are attempting to define the role of methylation reactions in the release of histamine from human basophils and mast cells. Methylation reactions mediated by S-adenosyl-L-methionine play an important role in a number of biological reactions including bacterial and human monocyte chemotaxis. Utilizing a number of inhibitors of methylation, strong evidence was found that methylation reactions play an important role in the early steps of IgE-mediated histamine secretion from human basophils. In studies with cultured mast cells, the methylation step was found to be due to the methylation of phospholipids in the membrane.

An active program has been initiated in the Clinical Immunology Section to develop a number of monoclonal hybridoma antibodies. The fusion of spleen cells from immunized mice with a myeloma cell line results in clones of cells producing antibodies to which the mice had previously been immunized. Utilizing this technology, monoclonal IgE hybridomas have been produced. Antibodies to cell surface Fc receptors have also been made and will be utilized in functional studies of these receptors. Antibodies of several clones have also been prepared to bacterial surface components and these can be utilized in studies of bacterial coaggregation as alluded to earlier. These monoclonal antibodies are, therefore, very useful reagents in a number of our immunological and microbiological studies.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE
PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF
HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DE 0045 08 LMI

PERIOD COVERED
October 1, 1978 - September 30, 1979

TITLE OF PROJECT (80 characters or less)
Macrophage and lymphocyte mediators that amplify T and B lymphocyte reactions

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.95	1.70	2.25

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)
Human macrophages when activated by a variety of agents produce lymphocyte activating factor (LAF). LAF exists in a 15,000 mw form which when mixed with serum changes to a 60,000 mw form. Both forms of LAF are mitogenic for peanut agglutinin negative (PNA$^-$) mouse thymocytes. The higher mw form also induces increased Lyt-1 expression by thymocytes and is mitogenic for human T lymphocytes. Only the lower mw replaces macrophages in lectin induced human T cell proliferation and enhances helper T cell dependent immunoglobulin production by human B cells. The mitogenic effects of LAF can be competitively inhibited by allose. Human T lymphocytes can be activated by lectins or antigens to produce a mitogenic factor(s) (LMF). LMF is also mitogenic for mouse PNA$^-$ thymocytes and elutes in the same two regions off Sephadex as LAF. LMF differs from LAF in that: 1)LMF production is inhibited by suppressor T cells 2)The 15,000 mw LMF is mitogenic for human B cells as well as for cytotoxic T lymphocytes 3)LMF is not inhibitable by allose. Presumably LAF and LMF amplify immunological reactions by promoting the growth and differentiation of distinct subpopulations of lymphoid target cells.

PHS-6040
(Rev. 10-76)

OBJECTIVES:

The role of mediators produced by human macrophages and lymphocytes in the afferent limb of the immune response is being investigated. These endogenous nonspecific mediators appear to be essential in activating and amplifying lymphocyte mediated immunological reactions. In addition, other investigators are observing that LAF is physicochemically inseparable from macrophage derived endogenous pyrogen and from the monocyte derived factor that stimulates "reticular" fibroblasts in the synovial tissues of inflamed joints to produce large amounts of prostaglandins and collagenase. Therefore LAF also appears to stimulate non-lymphoid cells in the hypothalamus and joints and may play a pivotal role in acute as well as chronic inflammatory reactions. Investigations of the nature and mechanism of action of these vital mediators in *in vitro* tissue culture models may provide the requisite understanding to permit eventual therapeutic manipulations of the signals initiating and augmenting inflammatory reactions.

METHODS:

1. LAF activity is operationally defined by its mitogenic effect on C3H/HeJ thymocytes. The effect of supernatants of human mononuclear cell, mouse peritoneal cell or fibroblast cultures or partially purified chromatography fractions of such supernatants in stimulating or enhancing thymocyte proliferation is determined.

2. Mediators such as LAF produced by macrophages and LMF produced by human lymphocytes are also assayed for their effects on:
   a) Thymocyte differentiation e.g., changes in expression of Lyt-1 and Lyt-2 markers on thymocytes using the fluoresence activated cell sorter (FACS).
   b) mitogenic effects on human circulating thymus derived (T) and bone marrow derived (B) lymphocytes.
   c) Enhancement of immunoglobulin production by human B cells.
   d) Intracellular levels of cAMP and cGMP.

3. In collaboration with Dr. Steven Mizel human LAF and LMF activities are being physicochemically characterized using Sephadex, sephacryl, phenyl sepharose, DEAE cellulose, hydroxylapatite and polyacrylamide gel (PAGE) chromatography.

MAJOR FINDINGS:

Agents that activate human macrophages to produce increased lymphocyte activation factor (LAF) activity were investigated. Projects that have been completed indicate that dialysable extracts of leucocytes which also contain transfer factor, the synthetic adjuvant muramyl dipeptide

(MDP), various extracts of mycobacterial cell walls, strains of corynebacterium parvum with adjuvant activity, the cocarcinogen phorbol myristic acetate (PMA) and the process of infecting mice with malaria all activate macrophage to produce LAF. Studies of serum factor(s) that activate macrophage LAF production have not been successful in identifying the responsible serum component and have been discontinued. The observation that murine colony stimulating factor(s) (CSF) induce macrophage LAF production is actively being investigated in collaboration with Dr. Robert Moore. CSF is a macrophage growth factor that is produced by either fibroblast cell lines or lectin stimulated lymphocytes. CSF stimulates not only macrophage proliferation, but also promotes their maturation so that they either produce LAF or become capable of responding to other agents that induce LAF production.

A multiplicity of effects of LAF on lymphoid cells is being studied:
1) In collaboration with Dr. Bonnie Mathieson (NIAID), we are finding that LAF is directly mitogenic only for the minor subpopulation of thymocytes that is not agglutinated with peanut agglutinin (PNA$^-$). The PNA$^-$ sub-population is presumed to comprise the medullary cortisone resistant thymocytes. In contrast the majority of PNA$^+$ (presumably cortical) thymocytes requires two signals to be in induced to proliferate. PHA and LAF together synergistically stimulate PNA$^+$ thymocytes, but have no effect not by themselves.

2) Studies in conjunction with Dr. Northoff of a battery of sugars have revealed that allose competitively and reversibly inhibits LAF induced thymocyte proliferation. This clue as to the nature of receptors for LAF is being used to prepare affinity columns for further purification of LAF.

3) The fluorescence activated cell sorter (FACS) is being used by Dr. Mathieson to detect changes in the surface markers of thymocytes incubated with LAF containing fractions. We are observing a dramatic increase in the expression of Lyt-1 and decrease in Lyt-2 on both PNA$^-$ and PNA$^+$ thymocytes after overnight incubation. The relationship of the factor that induces this thymocyte differentiation to LAF is being ascertained.

4) Dr. Douglas Olson (Meloy Labs) has been assaying cyclic nucleotides levels in lymphocytes incubated by us with a wide variety of stimulants. These studies have revealed that: (a) Although LAF containing supernatants stimulate considerable increases in thymocyte cAMP levels, this was actually due to prostaglandins rather than the LAF present in such supernatants. (b) Even though human LAF containing supernatants also stimulate rapid and dramatic increases in the cGMP levels of human mononuclear cells, this was actually due to a serum component(s) rather than due to the LAF present in the supernatants. (c) A study by L. Raupp with L. Lum and M. Blaese (NCI) has revealed that the cAMP levels of

human T cell subpopulations with Fc receptors for IgG (Tγ) are raised to a much greater degree by a variety of agents than the levels in the larger subopulations that bears Fc receptors for IgM (Tμ).

5) Partial purification of human LAF by sephadex chromatography yields two peaks of activity in the 15,000 and 60,000 mw range. The lower mw LAF when mixed with human serum is partially converted to the higher mw form of LAF. The higher mw human LAF is mitogenic for human peripheral blood T but not B lymphocytes. However, it can not replace the requirement for viable macrophages in antigen induced T cell proliferation. LAF therefore functions as a secondary amplifying factor rather than as an initiator of lymphocyte activation.

6) In collaboration with Dr. F. GMelig-Meyling, we found that only the low mw human LAF can substitute for macrophages in facilitating pokeweed mitogen (PWM) induced polyclonal immunoglobulin production by human B lymphocytes. Since PWM is T cell dependent, the low mw LAF presumably promotes T cell helper functions.

We have observed with Dr. G. Schechter (VA Hosptial) that the macrophages from anergic patients with Hodgkins disease are abnormal in their _in vitro_ LAF production. They produce inhibitors including prostaglandins However, the relationship of these observations to the disease process are as yet unresolved.

We are also studying lymphocyte derived mitogenic factors (LMF) produced by concanavalin-A (Con-A) and antigen (Streptolysin-0, SLO) activated lymphocytes. LMF is similar to LAF in that it stimulates PNA^- thymocyte proliferation and is eluted in the 60,000 and 15,000 mw regions from Sephadex. Both LMF and LAF are destroyed by chymotrypsin and SDS. However, LMF exhibits a number of differences from LAF: (1) Its production by human T lymphocytes is macrophage dependent. Its production especially in response to Con A is inhibited by T suppressor cells. Therefore the production of LMF can be enhanced by inhibiting suppressor cell functions with prolonged incubation or drugs (e.g.. cymetidine). (2) LMF is less susceptible to the inhibitory effect of allose than LAF. (3) Unlike LAF, both the higher and lower mw LMF are mitogenic for human peripheral T lymphocytes. In addition, the lower mw LMF (unlike LAF) is mitogenic for human B lymphocytes and is also capable of supporting the growth of cytotoxic T lymphocyte lines (CTLL).

SIGNIFICANCE TO DENTAL RESEARCH:

The central role of cell mediated and humoral immunity in periodontal disease has been documented. Macrophages as well as lymphocytes and plasma cells participate in chronic gingival inflammation. _In vitro_ tissue culture studies can be used to dissect the cellular interactions and to study the exogenous and endogenous signals that contribute to this inflammatory state. Thus, both unfractionated gingival plaque and cell wall extracts of Actinomycoses viscosus are potent activators of in vitro LAF production. This "monokine" may therefore augment inflammation in periodontal disease. Studies of LAF and lymphocyte derived mitogenic factors may eventually provide opportunities for therapeutic intervention in inflammatory reactions. Elucidation of amplifying effects of endogenous mitogenic factors on immunological reactions may provide the keys to pharmacological intervention in inflammatory reactions of the host.

PROPOSED COURSE:

Studies designed to purify, isolate and characterize monocyte and lymphocyte derived mitogenic factors will be pursued. Attempts to produce antibodies to these mitogenic factors have been initiated. Hybridoma cell lines that produce antibody will be established. Such antibodies can be used to facilitate further purification of these factors using affinity columns, to determine the effects of inhibiting these mediators, and to develop sensitive radioimmunoassays for detecting and identifying the mediators. Agents and conditions that regulate the production of these amplification factors will be investigated. The mechanism of action of these mediators must be clarified.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 DE-00131-05 LMI

PERIOD COVERED
October 1, 1978 - September 30, 1979

TITLE OF PROJECT (80 characters or less)

Regulatory Role of Thymus-Derived Lymphocytes on the In Vitro Antibody Response

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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4.25	3.25	1.00

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Macrophages (MØ) and T cells produce a variety of soluble factors which modulate the induction of the antibody and cell-mediated immune responses. The biochemical relationship between the multiple factors, their mode-of-action, and conditions under which they are produced have been investigated. Interleukin 2 (IL 2), which augments plaque-forming cell (PFC) and cytotoxic T cell responses, has been shown to be T cell-derived although MØ-dependent. The MØ requirement during the production of IL 2 can be replaced by either phorb 1 myristic acetate or MØ culture supernatants. Interleukin 1 (IL 1) (LAF) has been shown to be 1) produced in vivo, 2) capable of replacing the requirement for MØ during the induction of primary T cell responses and 3) capable of synergizing with a late-acting T cell replacing factor (TRF) in the induction of the antibody response. TRF has been shown to be distinct from the principal component of IL 2 on the basis of differential sensitivity to pH 2.0 and partial separation by hydrophobic chromatography. Thus at least three distinct immunoenhancing factors (IL 1, IL 2, and TRF) have been partially delineated biochemically and functionally. Studies are currently in progress to attempt to elucidate the mechanism by which these factors function in concert to enhance B cell and T cell responses.

OBJECTIVES:

The principal objectives of this research project are: 1) to purify and characterize the antigen nonspecific lymphokines which modulate the activation of the humoral and cell-mediated immune responses and 2) to determine the mechanism-of-action of these soluble factors.

METHODS:

Standard in vitro lymphocyte culture methods for the induction and assay of antibody and cell-mediated immune responses were utilized including the enumeration of antibody forming cells by the Jerne hemolytic plaque-forming cell (PFC) assay and the ^{51}Cr release assay for quantitation of cytotoxic lymphocytes. Standard cell separation procedures were used to prepare purified preparations of B cells, T cells, and macrophages.

MAJOR FINDINGS:

On the Production of Thymocyte Mitogenic Factor (TMF) (Interleukin 2 IL 2): We have previously shown that Con A-stimulated mouse spleen cells produce a soluble factor (designated TMF (IL 2)) which exhibits an apparent molecular weight of approximately 35,000 and enhances thymocyte proliferation, antibody synthesis, and the induction of cytotoxic lymphocytes. We have now examined the interaction between macrophages (MO) and T cells in the production of IL 2 and found both cell types to be required. Thus, from these types of experiments it has been impossible to determine the cell-of-origin of the factor. However, a number of experiments have shown that the ability of purified T cells to produce IL 2 following stimulation with Con A is restored by the addition of nanogram amounts of phorbol myristic acetate (PMA). Although the mechanism by which PMA replaces the requirement for MO is unclear, the results clearly demonstrate that the cell-of-origin of IL 2 is the T cell. Recent experiments also indicate that the MO dependencey during the production of IL 2 can be replaced by supernatants from MO cultures. These results indicate that activation of T cells to the production of IL 2 requires at least on "positive" signal from the MO and that this signal is provided by a soluble product of the MO.

Further studies with PMA on unfractionated spleen cells indicate that the addition of 100 nanograms of PMA to Con A-stimulated spleen cell cultures enhances the production of IL 2 by 6 to 10 fold. This observation provides a significant advance in our efforts to obtain sufficient material to continue the purification and characterization studies.

On the in vivo production of Lymphocyte Activating Factor (LAF) (Interleukin 1, IL 1): It has previously been reported that BCG infected mice that are administered LPS release into their serum a PFC helper factor and a tumor necrotizing factor. The two factors have been reported to co-purify suggesting molecular identity. We also confirmed the presence of a tumoricidal activity in BCG/LPS serum which is presumably identical to tumor necrotizing factor. We therefore investigated the biochemical relationship between the tumoricidal (cytotoxic) factor and the PFC helper factor. Gel filtration of ammonium sulfate (40-60% saturation) precipitated serum in a high ionic strength buffer (1.6 M NaCl), resulted in a separation of the two bioactivities. The PFC helper factor eluted with an apparent molecular weight of 12,500 and exhibited the capacity to enhance thymocyte proliferation. Thus, by four criteria; 1) molecular weight, 2) biological activity, 3) inducing stimulant, and 4) presumed cell-of-origin (MO), this helper factor is presumably LAF (Il 1) and thus represents the first demonstration of the in vivo production of this mediator.

The serum derived cytotoxic factor exhibited an apparent molecular weight of 55,000-60,000. Further characterization of this activity showed the substance was heat stable (30 minutes at 56°C); the isoelectric point was found at pH 4.6. Cytotoxic activity was retained by DEAE-Sephacel in tris buffer (pH 7.1) and eluted with a salt gradient at 0.2 m NaCl. The cellular source of this cytotoxin presumably is the activated macrophage since a soluble tumor cytotoxic activity with similar biochemical characteristics can be obtained from cultured macrophages in vitro after LPS stimulation. Tumoricidal factor is released into the culture supernatant following stimulation with LPS by: 1) adherent peritoneal exudate cells from BCG-infected mice, 2) peritoneal exudate cells that had been grown in culture for 14 days in the presence of macrophage growth factor (L cell supernatant), and 3) PU5-1.8 monocyte-macrophage cell line. Thus this activity might represent a mediator of macrophage cytotoxicity for tumor cells.

On the characterization of T cell replacing factor (TRF): We have previously shown that LAF (IL 1) enhances the antibody response of T cell-deficient mouse spleen cells in vitro and that it functions synergistically with a T cell-derived factor (TRF) to enhance the PFC response. We have now extended these studies in an effort to determine: 1) the optimal sequence of application of IL 1 and TRF in order to achieve maximal synergism and 2) the biochemical relationship between TRF and IL 2.

Although synergism was obtained when IL 1 and TRF-containing supernatants were added together to B cell cultures at the beginning of the culture period, maximal PFC responses were obtained when the addition of TRF-containing supernatnat was delayed by as much as 48 hr. In contrast, delayed addition of IL 1 resulted in a decreased PFC count. These results suggest that IL 1 and TRF function sequentially in the activation of the B cell response.

Recently we have achieved what appears to be a partial separation of IL 2 from the late-acting TRF using phenyl sepharose hydrophobic chromatography. Although the fraction pool containing the maximal amount of IL 2 synergized with IL 1, maximal synergizing late-acting TRF activity was present in a pool of fractions which contained significantly less IL 2. Further results indicate that IL 2 is resistant to pH 2.0 inactivation whereas at least a portion of the late-acting synergizing TRF activity is sensitive to pH 2.0. Thus, on the basis of functional and biochemical evidence, we have concluded that IL 2 and late-acting TRF are distinct molecular entities. The question as to the relationship between TRF and other lymphokines is under investigation. In this regard, the pH 2.0-sensitive TRF was found to elute from phenyl sepharose in fractions rich in immune interferon.

Finally, another factor which is neither IL 2, TRF, or interferon has been separated on phenyl sepharose. This latter factor(s) exhibits no intrinsic PFC helper activity and does not synergize with IL 1, but does enhance the synergism that one observes between IL 1 and TRF. The identity of this factor is currently under investigation.

On the role of IL 1 in the induction of cytotoxic lymphocytes: Macrophage culture supernatants were biochemically analyzed for factor(s) that restored the macrophage dependency of alloantigen specific cytotoxic T lymphocyte development. Throughout a sequential chromatographic purification scheme (i.e.gelfiltration, DEAE cellulose, and PAGE) factors that restored the macrophage function in killer cell development also co-eluted with the factor that stimulated thymocyte proliferation. Based on the cellular source (macrophages), molecular weight (approximately 15,000), charge heterogeneity exhibited on DEAE cellulose and polyacrylamide electrophoreses, and thymocyte mitogenic activity, the factor substituting for macrophages in the development of killer T lymphocytes was identical to IL 1 (LAF).

SIGNIFICANCE TO BIOMEDICAL RESEARCH:

The inflammatory response is regulated by a complex multicomponent network which includes as its active components antigen, antibody, complement, macrophages, helper and suppressor T cells, and B cells. These regulatory components serve to control the initiation, expression, maintenance, and suppression of the inflammatory response. Since periodontal disease is characterized by an inflammatory response within the gingiva and periodontium, an understanding of the regulatory mechanisms involved should allow us to intervene in the course of the inflammatory process. Since lympho-macrophate interaction is mediated in part by soluble factors produced by these cells and since the cellular interaction is a vital part of the regulatory network, the elucidation of the mechanism-of-action of the soluble factors is crucial to our understanding of inflammation.

PROPOSED COURSE:

In the succedding year experiments will be conducted in an effort to: 1) determine if IL 1 is capable of replacing the requirement for MO during the production of IL 2; 2) determine the target cell of both IL 1 and IL 2 in antibody synthesis and cytotoxic cell induction systems and 3) characterize and identify the nature of TRF and the co-factor which facilitates the synergism between TRF and IL 1.

PUBLICATIONS:

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 DE 00167-03 LMI

PERIOD COVERED
October 1, 1978 - September 30, 1979

TITLE OF PROJECT (80 characters or less)
The Biological Actions of Bacterial Endotoxin In vivo and In vitro.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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SUMMARY OF WORK (200 words or less - underline keywords)

The LPS-unresponsive C3H/HeJ mouse strain has been utilized to help elucidate the mechanism of action of bacterial endotoxin (LPS). These mice possess a defect in the LPS-responsiveness of their T and B lymphocytes, macrophages, and fibroblasts that is due to a mutation in a single, autosomal co-dominant gene. The biological effects of LPS are cell mediated because the interaction of LPS with lymphocytes and/or macrophages renders C3H/HeJ mice susceptible to LPS-induced lethality, immunogenicity, adjuvanticity, and tumor necrosis, as shown by the adoptive transfer of bone marrow cells from LPS-sensitive mice. C3H/HeJ mice can also be rendered LPS-sensitive by preinfection with BCG, a potent immunostimulant. Endotoxicity may be mediated by a number of LPS-induced acute phase reactants such as prostaglandins, lymphocyte activating factor, interferon, colony stimulating factor, and serum amyloid associated protein which are detectable in the sera of LPS-sensitive animals. The LPS gene may also play a vital physiological role since it appears to control the ability of macrophages to manifest LPS-induced tumor cell killing as well as the ability of macrophages to respond to LPS in vivo and in vitro with the production of physiologically relevant cytokines.

OBJECTIVES:

The major objectives of this project are: 1) to understand the mechanisms by which bacterial lipopolysaccharides and other related products produce lymphocyte and macrophage activation. 2) to evaluate the role that oral bacterial lipopolysaccharides play in the initiation and continuation of the chronic inflammatory process that results in periodontal disease.

MAJOR FINDINGS:

The C3H/HeJ mouse strain is unresponsive to all in the *in vivo* and *in vitro* effects of bacterial endotoxin (LPS). Our studies have concentrated on understanding the cause of LPS-unresponsiveness in this mouse strain and utilizing these mice as a model to elucidate the mechanism by which LPS induces its biological effects. We previously found that the B lymphocytes of these mice were intrinsically unresponsive to LPS. Furthermore, we found that it was possible to render these mice susceptible to the lethal effects of LPS by the adoptive transfer of spleen cells from a histocompatible but LPS-responsive mouse (C3H/HeN). This finding strongly suggested that endotoxicity was being mediated by an effect of LPS on some lymphoid cell. Our efforts have focussed on determining the type of lymphoid cells involved in mediating endotoxicity, and trying to determine the mechanisms by which this occurred. Our major findings have been:

1) The macrophages of C3H/HeJ mice were found to be intrinsically refractory to LPS as judged by a failure to respond to LPS *in vitro* with the production of lymphocyte activating factor or prostaglandins. These cells are additionally unresponsive to the direct cytotoxic action of LPS on macrophages. A formal genetic study was completed which demonstrated that macrophage LPS-sensitivity was controlled by a single, autosomal codominant gene that either was closely linked or identical to the gene controlling Bcell LPS responsiveness. In contrast, CBA/N mice, which possess a selective defect in B-cell LPS responsiveness, had normally LPS-sensitive macrophages. Since these mice are killed normally by LPS. these findings suggest that LPS-sensitive macrophages and not B cells are necessary for endotoxicity.

2) Additionally, several other studies have been completed which strengthen the role of macrophages as one of the important cells in mediating endotoxicity. First, we have established optimum conditions for the restoration of LPS-responsiveness in lethally irradiated C3H/HeJ mice by the transfer of LPS-sensitive C3H/HeN bone marrow or spleen cells. Reconstituted C3H/HeJ chimeras exhibit LPS responsiveness as measured by lethality, immunogenicity, adjuvanticity, and tumor necrosis, as well as by the production of certain LPS-induced acute phase reactants such as interferon, colony stimulating factor, and serum amyloid associated protein (SAA). Second, we have demonstrated that serum derived from LPS-injected C3H/HeN mice restored to the LPS unresponsive C3H/HeJ mice

the ability to produce serum amyloid associated protein. This serum factor appears to be a macrophage product since LPS-stimulated C3H/HeN macrophage culture supernatants also restored to C3H/HeJ mice the ability to produce SAA. These findings strengthen the hypothesis that many of the effects of endotoxin are mediated by the initial action of LPS on some lymphoid cell, presumably the macrophage.

3) Another approach has been taken to elucidate the cellular interactions involved in mediating endotoxicity. Infection of C3H/HeJ mice with BCG prior to LPS administration rendered them LPS sensitive as measured in vivo by LPS-induced lethality and hypoglycemia, as well as the production of serum interferon and SAA. In contrast, BCG-infection increased anti-LPS antibody production only minimally. The ability of BCG to induce LPS sensitivity in C3H/HeJ mice demonstrates that LPS responsiveness in C3H/HeJ mice is not due to an absolute defect in this strain, but rather, a partially reversible state of hyporesponsiveness. These findings also suggest that BCG infection acts primarily to enhance the LPS sensitivity of T cells and/or macrophages rather than B lymphocytes. Since, BCG has been shown to be a potent macrophage activating agent and appears to exert its action through T lymphocytes, studies are ongoing to determine the role of T cell-macrophage interaction in mediating endotoxicity. We have found that if one incubates LPS-unresponsive C3H/HeJ macrophages in the presence of a concanavalin A-stimulated spleen cell supernatant (CS) they will produce lymphocyte activating factor in response to LPS, suggesting that a T cell derived product can render an LPS-unresponsive macrophage, LPS-responsive. Furthermore, purified T cells from LPS responsive mice (including BCG-infected C3H/HeJ mice), cocultured with C3H/HeJ macrophages plus LPS, will produce a culture supernatant capable of causing thymocyte proliferation. These data strongly implicate T cell-macrophage interaction in endotoxin responsiveness. Finally, work has been completed which clearly demonstrates that in vitro LPS-induced adjuvanticity can be restored in LPS unresponsive B cell cultures by coculture with responder T cells and responder macrophages. Taken collectively, these data support the notion that T-macrophage interaction is essential for both the humoral and cellular manifestations of LPS sensitivity. In collaboration with Dr. Carl Hansen, we have characterized a potentially useful new mouse strain, the C57Bl/10ScN (nu/nu). We found that this athymic mouse strain was also LPS unresponsive and behaved comparably to the C3H/HeJ mouse strain in vivo and in vitro. This strain may provide a powerful new tool in the further examination for T cell participation in the initiation of LPS-induced tissue damage.

4) In addition to the LPS defect exhibited by C3H/HeJ macrophages, these cells lose, upon culturing, the ability to bind and phagocytose opsonized erythrocytes. Increased phagocytic ability has long been one measure of increased macrophage differentiation. This defect, like the

LPS defect, can be reversed by exposing the macrophages to a concanavalin A-stimulated spleen cell supernatant. Additionally, we have found that this defect can be reversed by dibutyryl-cyclic AMP. The relationship between the maintenance of Fc receptor capacity and LPS sensitivity will be actively pursued.

5) Since there appears to be an intimate relationship between macrophage LPS-sensitivity and macrophage activation, significant effort has been put forth to purify macrophage activating factor (MAF), a lymphokine capable of activating resident peritoneal macrophages to become tumoricidal or capable of killing intracellular parasites. The purification scheme employed results in a 75-fold purification and consists of an ammonium sulfate precipitation followed by chromatography on Sephadex G100 and DEAE cellulose. Purified murine MAF exhibits a molecular weight of approximately 55,000 daltons and exhibits charge heterogeneity. Purified MAF preparations will activate resident peritoneal macrophages to produce detectable levels of H_2O_2 and to cause the intracellular killing of the parasite Trypanosoma cruzi. In addition to characterizing the molecular nature of an extremely important lymphokine, these studies should help elucidate the relationship between LPS sensitivity and macrophage activation. Our studies to date strongly suggest a vital role for the LPS gene in maintaining host defense mechanisms and the homeostatic control of the immune system.

SIGNIFICANCE TO BIOMEDICAL RESEARCH:

Bacterial endotoxins are ubiquitous in nature. Because of their unique ability to activate B lymphocytes and macrophages, it is postulated that they may be essential to the normal maturation of the immune system. Furthermore, because of their diverse properties, they have been implicated as causative factors in certain pathological conditions such as gram negative shock. Our findings are therefore important to biomedical research for two reasons. First, they help us to understand the pathways by which B lymphocytes are induced to grow and divide. Secondly, they help us to understand the pathogenesis of the toxic effects of LPS, and suggest possible modes of therapeutic intervention.

One of the major goals of the NIDR is to understand the etiology of periodontal disease (PD) and to explore methods to prevent or eliminate this condition. Since PD is due to chronic inflammatory responses in the gingiva, and since endotoxins from oral flora are abundant in the mouth, it is important to understand how these endotoxins activate lymphoid cells to incite and prolong the immune response that results in PD.

PROPOSED COURSE:

1) The role of macrophages in mediating the effects of LPS will be

pursued. Our major thrust will be to evaluate the extent to which T lymphocyte-macrophage interaction underlies LPS sensitivity and how this responsiveness is related to macrophage activation. Having perfected our techniques for the adoptive transfer of lymphoid cells, we will now attempt to transfer specific cell populations such as macrophages and B and T lymphocyte populations to determine specific cellular requirements for LPS sensitivity. We will measure LPS sensitivity in vivo by measuring lethality, hypoglycemia, and the ability to produce anti-LPS antibodies in response to LPS and by measuring the appearance of interferon, CSF, or SAA in the serum following LPS administration.

2) We will try to determine the extent to which macrophage activation and LPS sensitivity are related, using Fc-receptor function as a marker for macrophage differentiation. We will pursue an analysis of the active agent(s) in concanavalin A stimulated spleen cell supernatant which corrects both the LPS defect and the Fc receptor defect in C3H/HeJ macrophages.

3) The role of specific macrophage products in manifestations of LPS sensitivity will be pursued. The potential role of LAF as the inducer of serum amyloid associated protein will be assessed.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 DE-00209-03 LMI

PERIOD COVERED
October 1, 1978 - September 30, 1979

TITLE OF PROJECT (80 characters or less)

Biological and Biochemical Characterization of Lymphocyte Activating Factor

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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	Ben-Zvi, Amos	Visiting Associate	LMI	NIDR
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2.25	1.75	.50

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The mouse macrophage cell line, $P388D_1$, in conjunction with the potent inflammatory and tumor promoting agent, phorbol myristic acetate (PMA) have been used to obtain sufficient quantities of lymphocyte activating factor (LAF) for purification and characterization studies. LAF has been purified 10,000-20,000 X with a yield of 7%. Chemical studies have revealed the importance of argenine groups in LAF activity. Biological studies have demonstrated that LAF enhances 1) thymocyte proliferation; 2) antibody synthesis in vitro; 3) the generation of alloantigen-specific cytotoxic T cells; 4) antigen-specific T cell proliferation; 5) the generation of stable E-rosette forming human T cells and 5) collagenase and prostaglandin production by rheumatoid synovial cells. Thus LAF may play an important, if not essential role in T cell activation for humoral and cell mediated immune responses as well as in inflammatory responses such as in the joints of patients with rheumatoid arthritis.

OBJECTIVES:

The major objectives of this ongoing project are: 1) to purify to homogeneity the macrophage-derived Lymphocyte Activating Factor (LAF) which appears to play an important, if not essential role in T lymphocyte activation; 2) to biochemically characterize the LAF; 3) to prepare a monospecific antiserum to the purified LAF, and 4) to characterize the spectrum of biological activities of LAF and the mechanisms that regulate LAF production.

MAJOR FINDINGS:

I. Biochemical Studies on LAF

We have continued to use the murine macrophage cell line, P388D$_1$, as a cell source of LAF and phorbol myristic acetate (PMA) as the stimulant for LAF production and secretion. The major findings are as follows:

1) Partially purified LAF was prepared by ammonium sulfate fractionation, and chromatography on DEAE cellulose, Sephacryl S200, and phenyl Sepharose, and electrophoresis in 10% Tris-glycinate discontinuous polyacrylamide gels. The increase in specific activity was approximately 10,000-20,000 fold with a yield of 7%. At a minimum, the LAF was 5% pure, being one of 13 proteins detected by two dimensional polyacryl amide gel electrophoresis and staining with a method sensitive to 0.1 ng protein/mm^2 gel. A projected specific activity of 1-3 x 10^7 units/mg protein was estimated for a purified preparation of LAF. Using preparative isoelectro focusing and high performance liquid chromatography we should soon approach this value. Of major importance is our ability to now associate a single protein band detected on the gels with the LAF biological activity. This is a new development in the field of lymphokines.

2) Injection of rabbits with highly purified LAF did not result in the production of active anti-LAF sera. We therefore examined the possibility that avians might be a more responsive species. We have recently obtained sera from chickens immunized with LAF in complete Freund's adjuvant. Although of relatively low potency (active $\geq$ 1/10 dilution) these sera exhibited some inhibitory activity on LAF-mediated thymocyte proliferation, whereas the proliferation in response to an unrelated T lymphocyte mitogenic factor was unaffected. We are therefore continuing to boost the chicken with LAF in an effort to increase the titer of the serum. The active material in this serum is not absorbed by the target thymocytes and copurifies with IgG.

3) Studies on the chemical nature of LAF have been continued. In previous studies, we demonstrated that LAF was relatively resistant to a number of endopeptidases, including chymotrypsin, papain, and trypsin; reduction and alkylation; and irreversible denaturation by urea and sodium dodecyl sulfate. These results were interpreted to indicate that LAF was a relatively inflexible polypeptide. In order to more precisely define the structural requirements for LAF activity, we initiated studies on the possible role of specific amino acids. LAF activity was decreased

in a time dependent manner by the argenine-specific modifying agent, phenyl glyoxal, indicating the functional importance of argenine in LAF activity. The essential arginine residues modified by phenyl glyoxal must be in the internal portion of the molecule, perhaps in the active site, since their presence on the external regions of LAF should have made them accessible to trypsin-mediated cleavage and LAF inactivation. Phenylglyoxal-treated LAF was not only less active as a stimulant for thymocyte proliferation, but was also less active in the induction of in vitro anti-sheep red blood cell plaque forming cell responses and the enhancement of rheumatoid synovial cell collagenase production (see project report on ACTIVATION OF RHEUMATOID SYNOVIAL CELLS BY LYMPHOCYTE ACTIVATING FACTOR).

In contrast to phenylglyoxal treatment of LAF, diethylpyrocarbonate modification of histidine residues resulted in only a 25% reduction in LAF activity, suggesting that histidine residues may play a less critical role in the action of LAF. Cyanogen bromide treatment of LAF did not generate any active fragments, suggesting that the active portion of LAF may be dependent on the total integrity of the molecule or alternatively, methionine groups are located in the active site of LAF.

II. Biological Studies on LAF Activity

In previous studies, we characterized the effects of $P388D_1$ cell derived LAF on thymocyte proliferation. In more recent studies, we have found that LAF may play a more general role in T lymphocyte activation and also in non lymphoid cells activation of the inflammatory response. Our major findings are as follows:

1) In conjunction with Drs. J. J. Farrar and M. L. Hilfiker of our laboratory we have found that LAF synergizes with several other lymphokines (at least two) in the induction of T cell-dependent in vitro antibody responses. The nature of these factors and their relation to LAF are currently being investigated.

2) The in vitro proliferation of primed murine lymph node lymphocytes (LNL) in response to the soluble antigen ovalbumin (OVA) was dependent upon the presence of peritoneal adherent cells. Restoration of OVA-induced LNL proliferation could be achieved by addition of highly purified LAF. LAF did not stimulate LNL proliferation in the absence of the priming antigen or T cells. Furthermore, treatment of LNL with anti-macrophage serum completely blocked the ability of these cells to respond to OVA + LAF, suggesting that the residual macrophages in the LNL population were necessary to provide an additional function or signal, possibly antigen presentation, in conjunction with LAF. These data therefore support the two signal hypothesis of macrophage-mediated lymphocyte activation and demonstrate the ability of LAF to provide one of these signals. Furthermore, these results indicate that one component of the amplifying effect of LAF on antigen-specific immune reactions may involve T cell proliferation.

3) In conjunction with Drs. W. F. Farrar and J. J. Farrar in our laboratory, we have found that LAF initiates the activation of alloantigen-specific

cytotoxic T cells. This effect, like that of LAF on T cell proliferation, is dependent on the presence of specific (allo) antigen.

4) Human T lymphocytes possess the capacity to form rosettes with red blood cells of a number of species, including sheep, ox, pigs, and guinea pigs. Two types of E-rosettes have been described: 1) those that occur at 4°C, but are unstable at 37°C and 2) those that are stable at both temperatures (stable E-rosettes). The number of stable E-rosette-forming T cells in the blood of normal patients is less than 5%. However, in acute lymphotic leukemia, chronic active hepatitis, and rheumatoid arthritis, the incidence of such cells may approach 60-70%. These cells possess the characteristics of activated T cells. We (A. Ben-Zvi, S. B. Mizel and J. J. Oppenheim) have found that LAF from the $P388D_1$ cell line or human mononuclear cells possesses the ability to induce the activation of stable E-rosette forming human T cells (as high as 60% of the T cell population). These findings indicate that LAF can function across species barriers and also that LAF might possibly be involved in the in vivo manifestation of these E-rosette forming cells. Supportive evidence for the possibility comes from studies on rheumatoid arthritis (see #5).

5) Isolated adherent synovial cells (ASC) obtained from rheumatoid synovectomy preparations produce large quantities of collagenase and prostaglandins in vitro. Although the precise cellular nature of the ASC is not well established, it is known that these cells do not possess the morphology or characteristic markers of macrophages or lymphocytes. The in vitro ability of ASC to produce collagenase and prostaglandins declines with time in culture, but can be restored by addition of a culture supernatant from human mononuclear cells. The active factor in the human mononuclear cell culture supernatant (mononuclear cell factor, MCF) was shown to be macrophage derived and of m.w. 10,000-20,000. MCF shares a number of biochemical properties with LAF. In view of the similarities between MCF and LAF, a collaborative project was initiated with Drs. Jean-Michel Dayer and Stephen M. Krane of Massachusetts General Hospital, Boston, Mass., to determine if MCF and LAF were biochemically and functionally related. We have found that MCF is also produced by the murine macrophage cell line, $P388D_1$. On the basis of its chemical properties and co-purification with LAF we have concluded that MCF and LAF are similar, if not identical molecular entities. Thus LAF can have effects on cell targets that are non-lymphoid in nature. The pathologic events in the joints of patients with rheumatoid arthritis may be associated with the infiltration of the synovial layers by various classes of cells, including macrophages, lymphocytes, and granulocytes. Analysis of the T lymphocytes from synovial fluid of arthritic patients has indicated that these cells are activated in terms of proliferation, stable E rosette forming capacity, and macrophage activating activity. In view of the ability of LAF to stimulate T cell proliferation and the generation of stable E rosette forming T cells, it is quite possible that macrophages in the synovium may regulate the inflammatory activity of both T lymphocytes and synovial cells by a single factor that is similar, if not identical to LAF. Recently, it was reported that collagenase production by stromal cells from injured rabbit cornea could be markedly

enhanced by a factor obtained from rabbit mononuclear cells. In view of our results, it is quite possible that this factor is also related to LAF. Thus LAF may play a general role in in vivo inflammatory reactions.

III. Studies on the Regulation of LAF Production

In previous studies, we demonstrated that the activation of LAF production and secretion by phorbol myristic acetate (PMA) a tumor promoter and inflammatory agent, involved the intracellular conversion of LAF from a high molecular weight, relatively inactive form to a low molecular weight form that was subsequently secreted by the macrophage. At present, Dr. Julianne Sando, a postdoctoral fellow who joined our laboratory in April, 1979, is engaged in studies on the precise mechanism(s) by which PMA activates the macrophage to release LAF. Using ^{3}H-PMA, we have established the presence of a binding receptors on P388D$_1$ cell line macrophages. The binding of ^{3}H-PMA to the target cells can be blocked by PMA-analogs in direct proportion to their tumor promoting and inflammatory activities. For example, the inactive tumor promoter, phorbol, did not inhibit ^{3}H-PMA binding, but phorbol didecanoate and phorbol dibenzoate which are active inflammatory agents did block ^{3}H-PMA binding to P388D$_1$ cells. These results indicate the pharmacological specificity of the ^{3}H-PMA binding. We are also examining the effect of PMA antagonists, such as retinoic acid, on PMA-induced increases in LAF production. Such compounds should provide additional tools with which to probe the fundamental mechanisms involved in macrophage activation.

SIGNIFICANCE TO BIOMEDICAL AND DENTAL RESEARCH:

Although it is well accepted that the immune system plays a dominant role in a variety of inflammatory reactions, the underlying mechanisms are not well understood. Several projects which have been undertaken in the LMI/NIDR, are united by a common effort to understand the role(s) of lymphokines in immunological function. It is only through an understanding of macrophage and lymphocyte function at the cellular and subcellular and molecular levels that we will be able to rationally manipulate the immune system for therapeutic purposes. In addition, the understanding of the mechanism(s) by which PMA, a potent inflammatory agent, activates macrophages would provide a foundation for a more general understanding of inflammation, especially as it applies to periodontal disease.

Our studies on the role of LAF in lymphocyte and synovial cell activation clearly raise the possibility that this factor may be a critical in vivo regulatory control mechanism in inflammation and immune reactions, both humoral and cellular.

The increasing attention given to lymphokines at international meetings, such as the Second International Lymphokines Conference at which I chaired a session on macrophage and lymphocyte activation, and in new journals, such as Lymphokine Reports (J. J. Oppenheim and I being members of the Editorial Board), is evidence of the increasing awareness of the importance of these substances in inflammation and human cell function.

PROPOSED COURSE:

1. Studies on the purification and characterization of LAF will be continued. We are currently selecting for constitutive mutants of $P388D_1$ cells that make increased amounts of LAF.

2. As purified LAF becomes available we plan to sequence the factor and possibly synthesize the active portion of the molecule.

3. Studies on the preparation of antisera will be continued using chickens as a source of serum.

4. Studies on the biological actions will be continued and extended. We intend to probe more deeply into the mechanism(s) by which LAF activates T cells in conjunction with antigen. In addition, we plan to examine the synovial fluid of patients with rheumatoid arthritis for the presence of LAF. The levels of LAF will then be correlated with the pathological situation in the joints.

5. The mechanism of action of PMA on LAF production will be studied in considerable detail. Binding studies with ^{3}H-PMA should lead to the isolation of the component(s) involved in the triggering of macrophage activation and LAF release.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 DE-00238-02 LMI

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)

Regulation of Mediator Production in Endotoxin-Stimulated Macrophages.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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2.20	1.95	.25

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The controlling mechanisms which regulate production of inflammatory mediators by endotoxin stimulated murine macrophages are currently under investigation. Research has concentrated upon four important mediators; interferon, granulocyte-macrophage colony stimulating factor (CSF), lymphocyte activating factor (LAF) and prostaglandins. Evidence obtained indicates that both prostaglandins and CSF may serve regulatory roles in mediator production by stimulated macrophages. E series prostaglandins (PGE) have been found to inhibit production of both CSF and interferon while CSF has been found to directly stimulate both PGE and LAF production by macrophages. Preliminary results suggest that CSF also sensitizes macrophages to endotoxin. CSF-cloned macrophages produced more interferon on a per cell basis than normal macrophages while normal macrophages preincubated with a source of CSF also responded in a hypersensitive manner. A partially purified CSF preparation has also been found to enhance LAF production by endotoxin-stimulated macrophages. Work in progress is designed to further investigate these opposing effects of PGE and CSF on macrophage functions.

OBJECTIVES:

The major objective of this project is to investigate the nature of regulatory mechanisms funtioning in an inflammatory response to control production of biologically active mediators. Particular emphasis is placed on understanding the interaction between macrophages and bacterial endotoxin resulting in production of these mediators.

MAJOR FINDINGS:

During the past year several aspects of macrophage responses to LPS have been investigated in *in vitro* culture systems. The results of these experiments are summarized later in this section. Several *in vivo* experiments, however, have also been performed which merit attention. Primarily these *in vivo* studies have focused on the cellular source of both CSF and interferon in endotoxin treated mice. Since macrophages have been shown to produce both mediators *in vitro* it has been assumed that these cells are a main source of both CSF and interferon *in vivo*. In our studies an attempt was made to validate these conclusions. For this purpose a system utilizing responder and nonresponder C3H mice was employed. The C3H/HeJ strain produced neither interferon or CSF when challenged with endotoxin while the C3H/HeN mice responded to endotoxin in normal fashion producing detectable serum levels of both mediators. Permanent chimeras were made between these mouse strains by a combination of lethal irradiation (850R) and bone marrow reconstitution (10^7 cells i.v), and after 4 weeks the chimeric mice were challenged with endotoxin. Briefly, the irradiated C3H/HeJ mice reconstituted with responder C3H/HeN bone marrow produced both CSF and interferon. While these results do not conclusively prove that macrophages are the major source of interferon and CSF in endotoxemic mice, they clearly demonstrate that the lymphoreticular system controls the production of both factors in response to the toxin.

Definite progress has also been made in understanding regulatory mechanisms functioning to influence mediator production by endotoxin-stimulated macrophages. In general PGE has been found to be suppressive while CSF appears to augment macrophage functions. Results obtained with PGE are summarized as follows:

1) Addition of indomethacin (an inhibitor of prostaglandin synthesis) to resident peritoneal leukocytes and to their adherent subpopulations (approximately 85% macrophages) at the time of endotoxin challenge resulted in a marked enhancement of CSF production. Since indomethacin neither influenced the assay for CSF nor inhibited production of suppressive factors by endotoxin-treated cells, these results were taken to indicate that prostaglandins produced by endotoxin-stimulated cells suppress CSF production. This was confirmed by adding exogenous prostaglandins to endotoxin-stimulated cultures in the presence of indomethacin. PGE, but not $PGE_{2\alpha}$, reversed the enhancement of CSF production by indomethacin.

Since CSF functions to enhance clonal proliferation and differentiation of granulocyte-macrophage stem cells, these results suggest that PGE, by suppressing CSF production, may serve to limit the myelopoietic response elicited by infection and/or inflammation.

2) Physiological concentrations of PGE have also been found to inhibit interferon production by endotoxin-stimulated macrophages. As little as 10^{-8}M PGE_1 or PGE_2 inhibited interferon production by approximately 50%. Addition of indomethacin to endotoxin-stimulated macrophages tended to increase the interferon yield suggesting that endogenous PGE synthesis may also serve to limit interferon production as well as CSF.

As stated earlier, CSF unlike PGE has been found to be stimulatory rather than suppressive in these studies. Summaries of our results with CSF are discussed as follows:

1. Macrophages treated with L_{929} cell conditioned medium (LCM) and supernatant from concanavalin A (Con A) - stimulated spleen cells produced lymphocyte activating factor (LAF). Both of these conditioned media contained CSF and macrophage growth factor (MGF) activities. Treatment with anti-CSF antiserum inhibited LAF production stimulated by the LCM, suggesting that CSF was the agent responsible for inducing LAF. Molecular sieve chromatography of both the LCM and Con A spleen preparations resulted in elution profiles, indicating that CSF and MGF are identical and that CSF/MGF directly stimulates macrophages to produce LAF. This observation is the first to date demonstrating that CSF serves a direct afferent function in the immune response.

2. CSF rich LCM has been found not only to directly stimulate macrophage LAF production but also to augment LAF production by endotoxin-stimulated macrophages. This enhancement has been demonstrated with macrophages from normal mice, the PU-5, 1.8 macrophage cell line and also with C3H/HeJ mouse macrophages which are normally unresponsive to bacterial endotoxin. Although preliminary in nature, these results suggest that CSF enhances the response of murine macrophages to LPS.

3. The effect of bacterial endotoxin on CSF (MGF) induced macrophage proliferation has been investigated. Macrophage colony formation in the presence of LCM was significantly inhibited by bacterial endotoxin. This inhibition, however, was biphasic in nature, i.e., the inhibitory effect of endotoxin increased with increases in CSF dosage while small doses of LPS in the presence of minimal CSF tended to increase macrophage proliferation. The inhibitory effect of LPS on macrophage proliferation was not due to cytotoxicity and was not mediated by prostaglandins. Although it is impossible at present to relate these findings to physiological situations, these results (as in 2 above) suggest that CSF "sensitizes" macrophages to the effects of endotoxin.

4. The effect on CSF or interferon production by endotoxin stimulated macrophages has also been investigated. Unlike LAF, simultaneous

addition of LCM and endotoxin to macrophages tended to suppress interferon production. Preincubation of macrophages with LCM for 72 hr, however, significantly increased interferon production by these macrophages. These preliminary results again suggest that CSF sensitizes macrophages to endotoxin.

A similar system suggesting enhanced responsiveness of CSF treated macrophages has been utilized by treating LCM-cloned macrophages with endotoxin. Macrophages propagated with LCM CSF (MGF) were found to be exquisitely sensitive to bacterial endotoxin. On a per cell basis, these cells produced at least 10 times as much interferon in response to endotoxin as control exudate macrophages.

SIGNIFICANCE TO BIOMEDICAL RESEARCH:

Macrophages exposed to inflammatory stimuli such as bacterial endotoxin are known to produce substances which have profound effects on surrounding tissues and other cells of the immune system. The experiments described above represent an attempt to ascertain regulatory mechanisms functioning to control macrophage responses to endotoxin. In brief, two separate controlling factors have been tentatively identified. E prostaglandins have been found to limit macrophage CSF and interferon production while CSF has been shown to directly stimulate LAF production. Preliminary data suggesting that CSF enhances endotoxin responsiveness of macrophages have also been obtained. These observations may prove to be of particular importance since both PGE and CSF are products of inflammatory and immunological reactions. Tentatively CSF and PGE may act to regulate these responses by serving as either enhancing or limiting signals.

PROPOSED COURSE:

The roles of CSF and PGE as potential regulators of macrophage functions will be investigated. With regard to PGE, studies will be initiated to determine if the suppressive effect on CSF and interferon production is mediated by alterations in intracellular cyclic nucleotide levels. Preliminary observations regarding CSF activities will be investigated extensively in an effort to determine if the active endotoxin-enhancing principal in L cell conditioned medium is indeed CSF.

Since macrophages occupy a central role in virtually all immune responses, biochemically characterized CSF preparations will be utilized to determine if CSF, through its macrophage stimulating properties, can affect either the initiation or expression of immune responses in vitro. Experiments will also be designed to investigate a potential role for CSF in influencing macrophage-fibroblast interactions. Production of fibroblast activating factor and/or collagenase by CSF stimulated macrophages would suggest a cooperative amplication mechanism between macrophages and fibroblasts in wound healing and repair.

PUBLICATIONS:

1. McGhee, J. R., S. M. Michalek, R. N. Moore, S. E. , and D. L. Rosenstreich, 1979. Genetic control of *in vivo* sensitivity to lipopolysaccharide: Evidence for codominant inheritance. *J. Immunol.* 122: 2052.

2. Michalek, S. M., R. N. Moore, J. R. McGhee, D. L. Rosenstreich, and S. E. Mergenhagen. The primary role of lymphoreticular cells *J. Infect. Dis.* In press.

3. Oppenheim, J. J., R. N. Moore, F. G'Melig-Meyling, A. Togawa, S. Wahl, B. J. Mathieson, S. Dougherty, and C. Carter. Role of cytokine and endotoxin induced monokines in lymphocyte proliferation differentiation and immunolglobulin production. *In* Regulatory Role of Macrophages in Immunity (Ed. Unanue and Rosenthal), Academic Press, New York. In press.

4. Moore, R. N., R. Urbaschek, L. M. Wahl, and S. E. Mergenhagen. Prostaglandin regulation of colony stimulating factor production by lipopolysaccharide stimulated murine lymphocytes. *Infect. Immun.* In press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 DE-00242-02 LMI

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)
The Role of Oxygen-Derived Free Radical Compounds in Inflammation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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	J. J. Oppenheim	Medical Officer	LMI NIDR
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COOPERATING UNITS (if any)
G. Schechter, Veterans Administration Hospital, Washington, D. C.

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Laboratory of Microbiology and Immunology

SECTION
Cellular Immunology

INSTITUTE AND LOCATION
NIDR, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.70	2.0	0

CHECK APPROPRIATE BOX(ES)
☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We have defined some of the effects of oxygen-derived free radical compounds (ODFRC) (viz, superoxide, hydroxyl, singlet oxygen and hydrogen peroxide) in inflammation by using three in vitro models of inflammatory cellular interactions. We have shown that the radical scavenger, reduced glutathione is a major positive determinant of the ability of murine spleen cells to produced an antibody response, in vitro. We have found that the enhancement of the antibody response by 2-mercaptoethanol is also mediated by glutathione. These enhancement effects can be mimicked by the addition of other known scavengers of ODFRC. Macrophages, stimulated in vivo, can suppress lectin-induced lymphocyte proliferation and serum-induced fibroblast proliferation. The use of inhibitors of prostaglandin synthesis and scavengers of ODFRC has provided evidence that the suppression of lymphocyte proliferation by macrophages is produced by a synergy of prostaglandins and ODFRC; similar experiments have provided evidence that the suppression of fibroblast proliferation by macrophages is mediated by ODFRC alone. Thus ODFRC appear to be important in the modulation of inflammatory cellular interactions.

PHS-6040
(Rev. 10-76)

OBJECTIVES:

1. Demonstrate the deleterious effects on mammalian cells, in vitro of spontaneously generated ODFRC.

2. Delineate the interactions of prostaglandins and ODFRC in inflammation model systems, in vitro.

3. Define both effective stimulants for the production of and scavengers against the effects of ODFRC.

4. Modulate the negative effects of ODFRC by the use of agents which either neutralize the activity of or stimulate the production of ODFRC.

METHODS EMPLOYED:

1. In vitro model systems:

   a) Primary antibody response of murine spleen cells, as described by Schreier and Nordin.

   b. Lectin-induced proliferation response of murine spleen cells, as initiated by concanavalin A.

   c. Serum-induced proliferation of mouse dermal fibroblasts, as described by Marcelo et al.

2. Assay for glutathione: the spectrofluorometric methods of Hissin and Hilf were used.

3. Stimulation, in vivo, of suppressive macrophages: intraperitoneal injection of heat killed bacterial cells (primarily Corynebacterium parvum) or thioglycolate broth.

4. Assay of primary antibody response: Cultured cells were collected and washed. The number of antigen-specific antibody forming cells was determined by the plaque-forming cell assay of Jerne and Nordin.

5. Assay of lymphocyte/fibroblast proliferation response: Cultured cells were pulsed with tritiated thymidine six hours (lymphocytes) or twelve hours (fibroblasts) prior to termination of the culture period. The cells were collected on fiberglass filters and the incorporated radiolabel was determined by a scintillation counter.

MAJOR FINDINGS:

Macrophages and polymorphoneuclear leukocytes produce excessive quantitites of oxygen-derived free radical compounds (ODFRC) as a mechanism of killing phagocytosed cells. Both in the normal respiring cell and in the stimulated phagocyte, the probable self-destructive effect of ODFRC is prevented by endogenous scavenging mechanisms (glutathione redox cycle, superoxide dismutase, catalase, α tocopherol, transferrin, caeruloplasmin, etc.).

The primary antibody response is apparently the most sensitive in vitro model which we have chosen to use, since an effect on any of the requisite complex cellular interactions will diminish antibody production. A critical variable in this system is the particular commercial lot of fetal calf serum (FCS) used in the culture medium. Using a quantitatively superior culture system, we have confirmed and extended our earlier findings concerning the function of the radical scavenging glutathione redox cycle in this model. We have shown that the reduced glutathione (GSH) content of FCS is a major determinant of the antibody response of murine spleen cells, in vitro. Furthermore, the enhancement of this antibody response by 2-mercaptoethanol (2-ME) is mediated by GSH in FCS. We have proposed that the action of GSH in this system is the protection of the responding cells from endogenously-produced ODFRC and from autoxidation. In support of that proposal, we have shown that specific scavengers of ODFRC can mimic the GSH-mediated enhancement effect and can act additively with suboptimal doses of 2-ME.

The intraperitoneal injection of heat-killed bacterial cells or thioglycolate stimulates the murine macrophage population to become "activated". These activated cells produce copious amounts of prostaglandins and ODFRC, as well as demonstrating many other enhanced metabolic functions. These cells are also capable of suppressing the lectin-induced proliferation of lympchoytes. We have confirmed the findings of others that prostaglandins contribute to this suppression, but we have also found that radical scavengers and inhibitors of prostaglandin synthesis act synergistically to protect against suppression of lymphocyte proliferation. We are now defining the functional interrelationships of ODFRC and prostaglandins.

In the simplest in vitro model, serum-induced fibroblast proliferation activated macrophages can also suppress proliferation. We have found that this suppression differs from the suppression of lymphocytes in that fibroblast suppression cannot be prevented by inhibitors of prostaglandin synthesis but rather, can be prevented by scavengers of ODFRC.

In Hodgkin's disease of humans, lectin-induced lymphocyte proliferation is suppressed. This suppression is attributable to the macrophages in the responding cell population. In some cases, prostaglandin production by these macrophages causes the suppression of lymphocyte proliferation. In collaboration with Dr. Geraldine Schechter, we have found that in certain cases, the suppression is attributable to ODFRC.

In collaboration with Dr. Philip Fox, we have been investigating the possible involvement of suppressive macrophages in human periodontal diseases. We have some early indications that such macrophages are present in some cases.

SIGNIFICANCE TO BIOMEDICAL AND DENTAL RESEARCH:

The interaction of responding immune cells with each other or with neighboring cell types has been shown to involve a number of relatively long-lived, soluble mediators (lymphokines, monokines, stable prostaglandins). The involvement of relatively short-lived mediators (ODFRC, thromboxanes, prostaglandin endoperoxides, prostacyclin) has only recently become apparent. ODFRC have been implicated in such *in vivo* conditions as rheumatoid arthritis and hyperuricemic arthritis as well as *in vitro* models of killing by immune cells. These mediators can not only affect surrounding cell types, but also can modulate the activity of other responding cells. Thus, an understanding of the mechanisms whereby ODFRC interact with various cells holds the promise of modulation of both immune reactivity and tissue damage in chronic inflammatory conditions such as periodontal disease.

PROPOSED COURSE:

We propose to continue these studies in several different ways:
1) The chemiluminescence assay permits direct time-dependent measurement of the production and accumulation of ODFRC. The use of this assay will allow us to screen stimulatory and scavenging compounds for their effects on the generation of ODFRC by various cell types.

2. We will investigate the possibility that ODFRC might also be short-lived positive signals.

3. We will continue the ongoing studies of the possible role of activated macrophages in the pathological processes of Hodgkin's disease and periodontal diseases.

PUBLICATIONS:

1. J. T. Hoffeld and J. J. Farrar. 1979. The characteristics functions, and interactions of macrophages, T cells, and B cells in the humoral immune response. *Dental Microbiology*. J. McGhee and S. Michalek (eds.). J. B. Lippincott, New York. In press.

2. J. T. Hoffeld and J. J. Oppenheim. 1980. The capacity of fetal calf serum to support a primary antibody response *in vitro* is determined, in part, by its reduced glutathione content. Cellular Immunology (In press).

3. Metzger,Z., J.T.Hoffeld and J.J.Oppenheim. 1980. Macrophage-mediated suppression: I. Evidence for participation of both hydrogen peroxide and prostaglandins in suppression of murine lymphocyte proliferation. Journal of Immunology (In press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 DE-00259-01 LMI

PERIOD COVERED

October 1, 1978 - September 30, 1979

TITLE OF PROJECT (80 characters or less)

Tumor Cell Derived Immunosuppressive Factors

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Mizel, Steven B.	Senior Staff Fellow	LMI	NIDR
OTHER:	Mizel, Diane	Chemist	LMI	NIDR
	Farrar, William F.	Post-doctoral Fellow	LMI	NIDR
	Hilfiker, Mary L.	Post-doctoral Fellow	LMI	NIDR

COOPERATING UNITS (if any)

George J. Todaro, National Cancer Institute
Joseph E. DeLarco, National Cancer Institute

LAB/BRANCH

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SECTION

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.25	.75	.50

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Murine sarcoma virus (MuSV) transformed mouse fibroblasts produce potent in vitro immunosuppressive factors (ISF). The partially purified ISF inhibited phytohemagglutinin (PHA) and concanavalin A (Con A) induced thymocyte proliferation, lipopolysaccharide (LPS)-induced spleen cell proliferation, the in vitro splenic anti-sheep red blood cell plaque forming cell response, and the generation of alloantigen specific cytotoxic T cells. The effect of ISF on thymocyte proliferation was not readily reversible and required only a 4 hr exposure of the thymocytes to ISF in order to inhibit cell proliferation. Although ISF shared several biochemical properties with a MuSV-transformed cell-derived sarcoma growth factor (SGF), e.g., acetic acid solubility, sensitivity to dithiothreitol, the two factors could be resolved by gel filtration on Bio-Gel P-60. Two peaks of ISF activity were found with apparent molecular weights of 12,000 and 8,000.

PHS-6040
(Rev. 10-76)

OBJECTIVES:

The major objectives of this new project are 1) to purify and characterize the immunosuppressive factors (ISF) produced in vitro by murine sarcoma virus (MuSV)-transformed mouse fibroblasts, 2) to evaluate their cellular targets, both in vitro and in vivo, and 3) to manipulate in vivo immunological activity.

MAJOR FINDINGS:

1) Murine sarcoma virus (MuSV) transformed mouse fibroblasts produce potent in vitro immunosuppressive factors (ISF). The partially purified ISF inhibited concanavalin A and phytohemagglutinin and lymphocyte activating factor-induced thymocyte proliferation, lipopolysaccharide-induced spleen cell proliferation, the in vitro splenic anti-sheep blood cell plaque-forming cell response, and the generation of alloantigen specific cytotoxic T cells. The effect of ISF on thymocyte proliferation was not readily reversible and required only a 4 hr exposure of the thymocytes to ISF in order to inhibit cell proliferation.

2) Preliminary studies on the in vivo activity of ISF indicate that ISF given to mice i.p. or i.v. can inhibit the mitogen responsiveness of spleen cells tested in vitro 2-3 days later.

3) Although ISF shared several biochemical properties with a MuSV-transformed cell-derived sarcoma growth factor (SGF), e.g., acetic acid solubility and sensitivity to dethiothreitol, the two factors could be resolved by gel filtration on Bio-Gel P60. Two peaks of ISF activity were found with molecular weights of 8,000 and 12,000.

SIGNIFICANCE TO BIOMEDICAL AND DENTAL RESEARCH:

Immunosuppressive factors have been described in the serum of tumor bearing humans and animals. The results of our studies have lead us to the conclusion that at least some of the immunosuppressive factors (ISF) may be actively produced by the tumor cells themselves. The study of these factors is therefore of some importance since such factors would obviously contribute to the in vivo survival of tumor cells. However, another important aspect of such factors is their use (theoretical at present) to regulate undesirable immune responses as, for example, in inflammation (periodontal disease) or autoimmune disease.

PROPOSED COURSE:

1) Studies will be continued on the purification and characterization of the ISF.

2) The cellular targets of ISF will be precisely defined.

3) In vivo studies with ISF in mice will be expanded to include the New Zealand Black strain of mouse which has been used as an experimental

model for the study of the immunopathology of autoimmune disease. Attempts will be made using ISF to modify the disease state.

PUBLICATIONS:

1. Mizel, S. B., J. E. LeLarco, G. J. Todaro, W. F. Farrar and M. L. Hilfiker. 1979. *In vitro* production of immunosuppressive factors (ISF) by murine sarcoma virus transformed mouse fibroblasts. Manuscript in preparation.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE
PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF
HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DE-00260-01 LMI

PERIOD COVERED

October 1, 1978 - September 30, 1979

TITLE OF PROJECT (80 characters or less)

Genetic Control of Immunologic Mechanisms

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Rosenstreich, David L.	Senior Surgeon	LMI	NIDR
COPI: Weinblatt, Anita	Post-doctoral Fellow	LMI	NIDR
OTHER: Gingras, Steven	Bio Lab Tech	LMI	NIDR

COOPERATING UNITS (if any)

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Dr. B. Taylor, Jackson Labs., Bar Harbor, Maine
Dr. M. Graves, Walter Reed Army Institute of Research, Washington, D. C.

LAB/BRANCH

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.25	.75	.50

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Natural resistance to a number of infectious disease organisms in mice is under genetic control. The nature of these genes and the immune mechanisms that they control are being investigated. Resistance to Rickettsia Tsusugomushi, the agent of scrub typhus, is controlled by a single, dominant gene (RIC) that is located in the middle of chromosome 5. This gene controls some function that is operative within four hours after bacterial infection. Resistance to Salmonella typhimurium, the agent of typhoid fever is controlled by three genes: 1) an x-linked gene that controls B cell differentiation (xio), 2) the LPS gene and 3) a chromosome 1 gene (Ity) that may control some aspect of cell mediated immunity. Resistance to Herpes simplex virus-1, the causative agent of acute gingivostomatitis and recurrent herpes labialis, is also polygenic. The number, location and mechanisms controlled by these genes are currently under investigation. Characterization of the genes that control resistance to infectious organisms will facilitate identification of individuals susceptible to any given disease and enable the development of appropriate preventive or therapeutic measures.

OBJECTIVES:

To identify and map the genes that control resistance to common infectious organisms in mice especially those such as Herpes simplex virus-1, that are involved in oral disease. To determine the mechanisms by which these genes control immunologic processes.

MAJOR FINDINGS:

Using recombinant inbred (RI) mouse strains and backcross linkage analyses, we have mapped a number of genes that control natural resistance.

1) R. Tsusugomushi. A single, autosomal dominant gene controls resistance to this organism. We have mapped this gene to chromosome 5, extremely close to the marker rd, and have designated this gene RIC with r and s representing the respective resistant and susceptible alleles. Mice are killed within 4 hours by very high numbers of this organism (toxic death). Susceptibility to toxic death is also controlled by RIC, indicating a genetic mechanism that functions very early after infection.

2) S. typhimurium. We have studied the role of 2 genes in resistance to this organism, Ity and LPS. LPS unresponsive mice are extremely susceptible to this organism, and backcross linkage analysis indicated that resistance was controlled by the LPS gene. Studies using adoptive transfer of cells or passive antibody, strongly suggest that this gene acts through a cellular (perhaps macrophage) mediated mechanism.

Using RI strains we have mapped the second gene Ity, to chromsome 1. Preliminary analysis suggests that this gene also controls resistance to another organism, the flagellate parasite, L. Donovoni.

3) Herpes simplex virus 1. Resistance to this organism has been previously found to be polygenic. RI strain analysis has confirmed this finding.

4) Other genes. We have also mapped another polymorphic gene locus of mice, SAS-1. This gene is located in the middle of chromosome 1. The function of this gene or its serum product remains unknown. However, it has proven to be an extremely useful marker for other genetic mapping studies.

SIGNIFICANCE TO BIOMEDICAL RESEARCH:

People vary tremendously in their susceptibility to infectious diseases including those caused by oral agents such as HSV-1 or the bacterial agents responsible for periodontal disease. The reasons for this variation in susceptibility are not known. Characterization of the genes that control resistance to infectious organisms will facilitate identification of individuals susceptible to any given disease and enable the development of appropriate preventive or therapeutic measures.

PROPOSED COURSE:

The mechanisms controlled by RIC, LPS and Ity will be investigated now that the genes have been mapped.

We will try to identify the exact number and location of the HSV-1 resistance genes. Mechanism studies will then be pursued.

PUBLICATIONS:

1. Rosenstreich, D. L., M. G. Groves, H. A. Hoffman and B. A. Taylor. Location of the SAS-1 locus on mouse chromsome 1. Immunogenetics in press.

2. Rosenstreich, D. L., M. G. Groves, A. O'Brien and B. A. Taylor. Genetic control of natural resistance. Proceedings of the Dahlem Workshop (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 DE-00261-01 LMI

PERIOD COVERED
October 1, 1978 - September 30, 1979

TITLE OF PROJECT (80 characters or less)
Signal Requirements for Lymphocyte Activation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Rosenstreich, David L.	Senior Surgeon	LMI	NIDR
COPI: Weinblatt, Anita	Post-doctoral Fellow	LMI	NIDR
OTHER: Gingras, Steven	Bio Lab Tech	LMI	NIDR

COOPERATING UNITS (if any)

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.25	.75	.50

CHECK APPROPRIATE BOX(ES)
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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

T lymphocytes require macrophage help in order to proliferate in vitro in response to mitogens and antigens. The compound phorbol myristate acetate (PMA) will replace macrophages and enable T cells to proliferate in response to a mitogenic but not an antigenic stimulus. T cells primed with PMA will also proliferate in response to a soluble product derived from the culture of antigen pulsed macrophages. We are currently examining the product in the culture supernatant of antigen pulsed macrophages to see if it exhibits the theoretically predicted properties of "processed antigen"--i.e., antigen specificity and genetic restriction in its effects or if it is a non specific T-cell triggering molecule.

PHS-6040
(Rev. 10-76)

OBJECTIVES:

To understand the molecular signals required to activate T cells.

MAJOR FINDINGS:

T lymphocytes *in vitro* will not proliferate unless supplied with macrophage help; either in the form of an intact cell or with a soluble product derived from macrophages, T-cell activating factor (TAF). Macrophage accessory function for mitogenic activation can be replaced completely by PMA. PMA by itself is non-mitogenic for purified guinea pig T cells, and seems to act as a second activating signal.

PMA will not enable T cells to respond to specific antigens *in vitro*. This is presumably because of an additional requirement for a macrophage Ia molecule related signal that must be associated with the antigen. This additional molecule was produced by collecting the culture supernatant of macrophages that had been pulsed with specific antigen. This material is undetectable in culture supernatants or intracellularly until 2 hours of culture. It is maximal at 4-24 hours of culture.

SIGNIFICANCE TO BIOMEDICAL RESEARCH:

Activated T lymphocytes are an essential component of almost all immune reactions. Identification of the signals that are required to activate these cells will further our understanding of the immune process, and may lead to the development of pharmacologic agents specifically designed to enhance or suppress immunity.

PROPOSED COURSE:

1) Biochemical basis of lymphocyte activation. Using the two defined compounds that are required to activate T lymphocytes, PMA and a plant lectin, we will attempt to define the mechanisms underlying lymphocyte triggering. The effect of each agent will be examined individually or in combination, for its effects on biochemical pathways known to be involved in cell division--i.e. cyclic nucleotides, nuclear ornithine decarboxylase, membrane phospholipids.

2. Macrophage processed antigen. The product in the culture supernatant of antigen pulsed macrophages will be examined to see if it exhibits the theoretically predicted properties of "processed antigen"--i.e., antigen specificity and genetic restriction in its effects. Biochemical characterization will be performed to characterize and purify this material. Finally, this material will be analyzed in the manner proposed in part 1 to identify its mechanism of action.

PUBLICATIONS:

1. Rosenstreich, D. L. and S. M. Mizel. The participation of macrophages and macrophage cell lines in the activation of T-lymphocytes by mitogens. Immunological Reviews 40: 102-135, 1978.

2. Rosenstreich, D. L. and S. M. Mizel. Signal requirements for T-lymphocyte activation I. Replacement of macrophage function with phorbol myristic acetate. J. Immunol. in press.

3. Rosenstreich, D. L., A. Weinblatt and L. Weedon. The use of synthetic compounds to replace the macrophage requirement for T-lymphocyte activation. Proceedings of the Brook Lodge Workshop on Macrophage Function. ed., A. Rosenthal and E. R. Unanue, in press.

4. Rosenstreich, D. L. The macrophage In The macrophage in the cell biology of immunity and inflammation. J. J. Oppenheim, M. Potter and D. L. Rosenstreich eds., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 DE-00046-09 LMI

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)

Role of Lymphocyte and Macrophage Mediators in Inflammation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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OTHER:	Wahl, Larry M	Research Biologist	LMI NIDR
	McCarthy, James B.	Biologist	LMI NIDR
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COOPERATING UNITS (if any)

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LAB/BRANCH
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SECTION
Humoral Immunity

INSTITUTE AND LOCATION
NIDR / NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.20	1.45	.75

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Research in this laboratory focuses primarily on the role of lymphocytes and macrophages in the regulation of connective tissue metabolism associated with inflammatory reactions. Antigen specific activation of lymphocytes results in the production of biologically active lymphokines. One of these lymphokines has the ability to stimulate fibroblasts to divide and to increase their synthesis of both collagen and noncollagen protein. Another lymphokine, macrophage activation factor, activates macrophages. Macrophages also produce a molecule which enhances fibroblast function. Activation of macrophages by MAF and other stimulants involves prostaglandin generation and alterations in intracellular levels of cAMP. By these mechanisms the immune system may modulate connective tissue metabolism in inflammatory lesions.

PHS-6040
(Rev. 10-76)

OBJECTIVES:

The ongoing research in this laboratory focuses on understanding the mechanisms whereby the immune system may modulate connective tissue metabolism. Connective tissue metabolism is markedly altered in many inflammatory lesions. The co-existence of inflammatory cells and connective tissue cells in such lesions led us to investigate whether lymphocytes could influence fibroblast function.

In addition to lymphoid cells, macrophages play a prominent role in the immune response. We are investigating how lymphokines and other agents including the synthetic adjuvant, muramyl dipeptide, activate these phagocytic cells to migrate, to release biologically active monokines, and to increase their biochemical functions as primary perpetrators of the inflammatory response.

Another cell which becomes functionally modified in inflammatory lesions is the epithelial cell. Using gingival epithelial cells we are attempting to define those agents, both immunologically-derived and nonspecific, which may influence these cells to regenerate the epithelial surfaces after an interruption in their continuity occurs.

METHODS EMPLOYED:

Methods utilized to investigate the aforementioned objectives include already published procedures for macrophage chemotaxis, lymphocyte culture, proliferation, lymphokine production, prostaglandin assay, cyclic AMP and GMP determinations, fibroblast culture, analysis of collagen formation, and epithelial cell culture.

MAJOR FINDINGS:

Lymphocytes exposed to specific antigen respond by producing numerous effector molecules. One such recently described molecule, fibroblast activating factor (FAF), exerts its effect on quiescent fibroblasts by causing these cells to proliferate and to increase their synthesis of protein. T lymphocytes from dinitrophenylated ovalbumin (DNP-OA) immunized guinea pigs, when challenged with the antigen in vitro, produced significant amounts of this mediator within 24-48 hr. Addition of these supernatants to subconfluent cultures of guinea pig dermal fibroblasts in serum-free medium resulted in fibroblast proliferation as determined by tritiated thymidine uptake and by an increase in cell numbers. This lymphocyte derived mediator can also modulate other fibroblast activities. Within 46 hr after exposure to FAF, fibroblasts synthesized and released greatly enhanced levels of prostaglandins into their culture medium. FAF also induced elevations in intracellular cyclic nucleotide (cAMP) levels. A significant increase in protein synthesis of both the collagen and noncollagenous types occurred prior to the major peak of fibroblast proliferation.

In addition to T cells, guinea pig peritoneal macrophages stimulated with lipopolysaccharide or muramyl dipeptide produced a soluble factor which activated fibroblasts to proliferate. Stimulated macrophages, but not control cells, released this factor which was measurable early after activation (1-2 hr) and increased during the subsequent 2448 hrs. This factor is nondialyzable, heat stable and has a molecular weight of 40-60,000. Additionally, a small dialyzable molecule was found in these supernatants which inhibited thymidine uptake but not cell division. Further characterization of these molecules is in progress. It thus appears that both macrophages and lymphocytes when appropriately activated release soluble molecules that can regulate fibroblast function. These fibroblast activating factors may contribute to the fibrotic reactions associated with chronic inflammatory lesions.

Further investigation into the mechanisms of macrophage activation revealed that muramyl dipeptide, lipopolysaccharide and lymphocyte derived macrophage activating factor which induced fibroblast activating factor production also activated the macrophages to produce collagenase. Collagenase production was dependent upon an increase in prostaglandin synthesis which in turn elevated intracellular levels of cAMP. That this elevation in cyclic AMP may control macrophage activation will be further explored by determining its role in modulating the production of biologically active mediators such as the fibroblast activating factor.

In light of our findings that lymphocytes and macrophages can influence fibroblast function, we have explored an _in vivo_ model of delayed hypersensitivity hypersensitivity for possible interactions between lymphoid cells and fibroblasts. Infection of mice with _Schistosoma mansoni_ results in granuloma formation in the liver which is then followed by hepatic fibrosis. The cause of this fibrosis, which results in death of the animals, is unclear. Our investigations suggest that the liver granulomas which form in response to the schistosomal infection stimulate fibroblast proliferation with the release of a soluble molecule(s) and thus may play a role in the development of hepatic fibrosis in _S. mansoni_ infections. We are currently attempting to define the cellular source of this fibroblast stimulating mediator within the granuloma.

In related studies, the role of inflammatory cells on epithelial populations is being explored. Following injury epithelial cells alter their normal behavioral patterns and begin to migrate, to phagocytize and to divide. It appears that inflammatory leukocytes may be, in part, responsible for influencing these cells to divide through the transmission of a soluble signal(s). Further analysis of such a mechanism is underway.

SIGNIFICANCE TO BIOMEDICAL AND DENTAL RESEARCH:

Cellular immune phenomena are the basis for the development of many chronic inflammatory lesions. There are certain pathologic conditions including scleroderma, pulmonary fibrosis, and schistosomiasis which have been characterized as cell-mediated which lead to excessive connective tissue formation or fibrosis. It is possible that the changes in the connective tissue could be mediated by the local production and release of lymphokines by antigen activated lymphocytes. Lymphokines appear to regulate fibroblast function suggesting a pathway for enhanced connective tissue metabolism including collagen formation.

Chronic inflammatory lesions which are characterized by an infiltrate of mononuclear cells may also be destructive to the surrounding connective tissue as in rheumatoid arthritis and periodontal disease.

In connective tissue destruction, immunologic events may initiate the process with the ultimate degradation actually brought about by inflammatory cells such as the macrophage through the production and release of proteolytic and tissue degrading enzymes.

Activated lymphocytes may also be largely responsible for the production of chemotactic stimuli which attract macrophages to sites of infection or inflammation. Once localized at such a site these macrophages may be activated to produce a number of enzymes and to release soluble factors (prostaglandins, fibroblast activating factor etc.), which influence other cells. If continuously activated as in chronic inflammatory lesions like periodontal disease and rheumatoid arthritis, these cells may contribute to the pathologic tissue destruction by the release of lysosomal enzymes, collagenase, and prostaglandins. Thus, through an understanding of the way these cells are triggered, it may be possible to control or modulate their function.

Macrophages may also play a prominent role in enhancement of immunological events associated with the use of adjuvants. The adjuvant effects of mycobacteria can be replaced by chemically defined isolates of the cell walls including a water soluble fraction (WSA) and by the synthetic analog, N-acetyl-muramyl-L-alanyl-D-isoglutamine (MDP) which is the minimal structure required for adjuvanticity. These clearly defined compounds can directly activate macrophages suggesting that immunologic adjuvanticity is related to enhanced macrophage function. The activation of macrophages may then in turn precipitate a multiplicity of other actions resulting in enhanced immune responses.

Mechanisms regulating re-epithelialization following an inflammatory lesion have been undefined and little explored in the past. If inflammatory cells can regulate epithelial cell behavior, it may enable manipulation of this system to encourage or to delay the re-epithelization process to favor the resolution of certain pathologic situations.

PROPOSED COURSE:

Investigations are continuing into the mechanisms of induction of inflammation which in some cases may progress to chronic pathologic lesions. The pathways by which lymphocytes are triggered to proliferate and to release the factors responsible for mediating inflammatory lesions require further study. Areas of study will focus on the interaction between the lymphoid system and connective tissue components. Lymphocytes and macrophages appear to be able to influence fibroblast growth and thereby may provide a pathway for normal connective tissue reconstruction following injury and/or for the markedly altered connective tissue changes which occur in various pathologic conditions. These studies will be pursued both in our in vitro model and also in the in vivo model of schistosomiasis. Characterization of the soluble mediators produced by the lymphocytes and macrophages will be correlated with the mediators released from active granulomas. Additionally, the role of lymphocytes and monocytes in modulating fibroblast function in the pathophysiologic processes associated with scleroderma in humans will be investigated.

Attempts will be made to further define the various parameters of macrophage activation and how they relate to variations in the intracellular levels of 3'-5' cAMP. Such parameters include nonlysozomal enzyme release (e.g. collagenase), lysozomal enzyme release (e.g. lysozyme), phagocytosis, glucose uptake, protein synthesis and the production of fibroblast activating factor. It is essential to understand the process of macrophage activation in order to be able to control it in chronic inflammatory lesions.

Involvement of lymphocytes and macrophages in modifying epithelial cell function will be explored. The ability of epithelial cells to migrate, o proliferate, to phagocytize and to produce Type IV collagen will be studied. We will determine whether soluble mediators from antigen-activated lymphocytes and/or from activated macrophages can enhance these parameters of epithelial cell function.

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1. Wahl, S. M., L. M. Wahl, and J. B. McCarthy. 1978. Lymphocyte mediated activation of fibroblast proliferation and collagen production. J. Immunol. 121: 942.

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13. Wahl, S. M., L. M. Wahl and S. E. Mergenhagen. 1979. Lymphokine and monokine regulation of fibroblast function. In Biochemical Characterization of Lymphokines. Proc. of the Second. Intl. Lymphokine Workshop (A. L. DeWeck, ed.), Academic Press, In press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)

U.S. DEPARTMENT OF
HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DE-00061-06 LMI

PERIOD COVERED
October 1, 1978 - September 30, 1979

TITLE OF PROJECT (80 characters or less)
Complement Activation and Inflammation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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OTHER:	Wahl, Larry M.	Research Biologist	LMI	NIDR
	Pazoles, Pamela P.	Chemist	LMI	NIDR

COOPERATING UNITS (if any)
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Laboratory of Microbiology and Immunology

SECTION
Humoral Immunity Section

INSTITUTE AND LOCATION
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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.95	.95	1.00

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The mechanisms by which the complement system regulates the metabolism of connective tissue continue to be investigated. In organ cultures of fetal rat bones, complement activation initiated by antibodies reactive with cell surface antigens results in the enhanced synthesis of prostaglandins which, in turn, causes resorption of the bone. A cell similar to a monocyte may be responsible for the elevated levels of prostaglandins found in these cultures since complement activation by immunoglobulins stimulates prostaglandin production by macrophages in culture. Immunoglobulin fragments which are incapable of directly activating these cells but which activate the alternative complement pathway are effective in this regard. Following interaction of the components of the complement system with macrophage membranes, enhanced incorporation of prostaglandin precursors into prostaglandins occurs. These studies demonstrate that both steroid as well as non-steroid inhibitors of prostaglandin biosynthesis abrogate this response. Complement activation also inhibits the growth of bone in organ cultures of calvaria. Although prostaglandin production is stimulated in these cultures, this event is unrelated to bone growth since indomethacin inhibits prostaglandin production but does not restore bone growth.

Studies have been initiated to examine the possible role of the complement system in the production of other cellular mediators which influence connective tissue as well as definition of the complement components involved.

OBJECTIVES:

It is becoming apparent that connective tissue metabolism can be regulated by several immunological mechanisms, both cellular and humoral. Ongoing studies demonstrate that complement activation initiated by immunoglobulins causes the destruction of bone and that this effect is mediated by prostaglandins. Current emphasis is directed towards delineation of the interaction of complement components with cell membranes which results in stimulation of prostaglandin synthesis and the pharmacological modulation of this effect. The role of the complement system in inhibiting bone growth is also under investigation. Prostaglandins are apparently not involved in this process and attempts will be made to define the mechanism(s) by which complement activation is detrimental to the growth of bone, the complement components required and the possible alleviation of this effect. Other functions of the complement system which may influence connective tissue metabolism, such as mediator production, will be explored.

METHODS EMPLOYED:

Bone resorption is quantitatively assayed by determining the ^{45}Ca released into the media of fetal rat bone organ cultures. Prostaglandins are determined by specific radioimmunoassay. Bone growth is assayed by incorporation of ^{3}H proline into collagenase sensitive and collagenase resistant protein in rat calvaria in organ culture.

MAJOR FINDINGS:

Immunoglobulins reactive with cell surface antigens present in fetal rat bone initiate complement activation and resorption of the bone. This biological effect is mediated by synthesis of prostaglandins by a cell(s) present in the bone.

In order to more adequately define the mechanisms involved in this process the interaction of antibody and complement with the macrophage, a cell similar to that which has been implicated in bone resorption (the osteoclast), has been investigated. In the presence of normal rabbit serum and anti-rat erythrocyte immunoglobulins rat peritoneal macrophages produce markedly elevated levels of prostaglandin E when compared with macrophages cultured with antibody and heat inactivated rabbit serum. The late complement components, at least through C6, are required for this effect since C6 deficient rabbit serum does not support this reaction but addition of functionally purified C6 restores the activity. Immunoglobulins can bind to either specific antigens or Fc receptors on the macrophages.

To determine if macrophage activation requires both the interaction of complement with the macrophage membrane and binding of antibody to the Fc receptors, $F(ab')_2$ fragments were utilized. These immunoglobulin preparations which interact only with the cellular antigens and activate only the alternative complement pathway were as effective as intact antibody in initiating complement dependent prostaglandin synthesis by macrophages. Thus, the function of antibody in complement dependent prostaglandin synthesis by these cells appears to be only to initiate complement activation at a cell surface. Complement markedly enhanced the incorporation of the prostaglandin precursor into prostaglandins by macrophages treated with $F(ab')_2$ fragments.

Similar results were obtained in cultures of fetal rat bones in that in the presence of complement and arachidonic acid, prostaglandin levels were elevated and, in addition, ^{45}Ca release from the bones was significantly increased. These results suggest that complement alters the cell membrane sufficiently to allow access of the prostaglandin precursor to the enzymes involved in the biosynthesis of prostaglandins. These effects were abolished by the addition of the prostaglandin synthetase inhibitor, indomethacin.

Another non-steroid agent, flufenemic acid also inhibits the complement dependent enhancement of arachidonic acid stimulation of prostaglandin production and bone resorption. Numerous mechanisms have been postulated for the effects of the glucocorticoid, hydrocortisone, on prostaglandin synthesis. Its inhibition of the complement mediated elevation of prostaglandin levels as well bone resorption in cultures containing arachidonic acid suggests that one site of action of this pharmacological agent is the inhibition of the incorporation of prostaglandin precursors into prostaglandins.

Another effect of complement on bone metabolism has also been defined. In addition to its role in inducing bone destruction, complement also inhibits bone growth. Determination of the uptake of ^{3}H proline in organ cultures of rat calvaria has demonstrated that antibody activation of complement inhibits the synthesis of both collagen and non-collagen protein. This effect is also dependent on the late complement components, at least through C6. Although complement and antibody stimulate prostaglandin synthesis in rat calvaria no correlation exists between the production of this agent and inhibition of bone growth. Indomethacin and flufenamic acid inhibit the synthesis of prostaglandins but are completely ineffective in restoring bone growth in cultured rat calvaria. Thus, C may have a dual function in the regulation of bone metabolism: it not only induces resorption of bone but, in addition, inhibits the growth of bone.

SIGNIFICANCE:

In certain inflammatory lesions such as periodontal disease and rheumatoid arthritis, destruction of connective tissue is a common finding. Elevated levels of prostaglandins have been found both in inflamed gingivae and the synovia of patients with rheumatoid arthritis. Prostaglandins are known to be potent bone resorbing agents. Since complement activation has now been shown to result in enhanced synthesis of prostaglandins by bones in organ culture as well as by macrophages, the complement system is implicated as a contributing factor to the destruction of connective tissue. In addition antibody initiation of complement activation can also prevent the synthesis of new connective tissue elements.

PROPOSED COURSE:

Studies will be continued to investigate the mechanisms by which immunological activation of the complement system influences connective tissue metabolism. In investigations of both bone resorption and stimulation of prostaglandin synthesis in macrophage cultures isolated components of complement will be utilized to define their interaction with membranes. The specificity of the antibodies required for initiation of these complement dependent events will also be examined. Complement interaction with macrophages and other cells will also be examined to determine if this type of cellular stimulation can result in the production of mediators other than prostaglandins which influence connective tissue metabolism. A possible defect in prostaglandin production by complement stimulated macrophages from osteopetrotic mice will also be examined as will the effects of complement and antibody on resorption of bones from these animals and their normal littermates.

PUBLICATIONS:

1. Wahl, L. M., Olsen, C. E., Wahl, S. M., McCarthy, J. B., Sandberg, A. L. and Mergenhagen, S. E. Prostaglandin and cAMP regulation of macrophage involvement in connective tissue destruction. N. Y. Acad. Sci. 1979. In press.

2. McCarthy, J. B., Wahl, S. M., Rees, J., Olsen, C. E., Sandberg, A. L. and Wahl, L. M. Regulation of macrophage collagenase production by 3'-5' cyclic adenosine monophosphate. J. Immunol. 1979. In press.

Larry Wahl

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 DE 00216-03 LMI

PERIOD COVERED

October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)

Immunological Control of Connective Tissue Metabolism

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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	Sandberg, Ann L.	Research Biologist	LMI NIDR
	McCarthy, James	Microbiologist	LMI NIDR
	Winter, Christine	Microbiologist	LMI NIDR

COOPERATING UNITS (if any)

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Shigeto, Abe - LBDA

LAB/BRANCH

Microbiology and Immunology

SECTION

Humoral Immunity Section

INSTITUTE AND LOCATION

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.20	1.70	1.50

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this project is to study the role of the immune system in connective tissue metabolism. We have demonstrated that the prostaglandins control the production of collagenase by endotoxin and lymphokine activated macrophages by regulating the intracellular levels of cyclic nucleotides (cAMP). Additionally, drugs, such as β adrenergic stimulators, dibutyryl cAMP and phosphodiesterase inhibitors, which directly effect cAMP metabolism have been shown to modulate both prostaglandin and collagenase production of endotoxin activated macrophages. High concentrations of these cAMP enhancing agents significantly inhibit the production of prostaglandin and collagenase whereas both are stimulated by lower concentrations of these drugs. Studies involving the role of the immune system in bone resorption have revealed that macrophages from an osteopetrotic rat (toothless rat) model are defective in the production of prostaglandins and in the production of suppressors of lymphocyte proliferation. The lower production of prostaglandins by macrophages from the osteopetrotic rats may in part be one of causes for the hyperproliferation of lymphocytes in the animals since prostaglandins are known to suppress lymphocyte proliferation.

PHS-6040
(Rev. 10-76)

MAJOR FINDINGS:

We have previously shown that the prostaglandins (PG) produced by activated macrophages regulate subsequent production of collagenase by these cells. Our recent findings demonstrate that prostaglandins control macrophage collagenase production by directly modulating the intracellular levels of cyclic adenosine monophosphate. Macrophages activated by endotoxin or lymphokine began producing PG within 1 to 2 hr and reached a peak in production between 14-18 hr. Kinetics of PG and cAMP changes show that there is a significant elevation in cAMP which occurs subsequent to the appearance of prostaglandins and extends over a 20-24 hr period. The addition of indomethacin, a prostaglandin synthestase inhibitor, to the macrophage cultures blocked the increase in both prostaglandins and cAMP and the subsequent production of collagenase. The role of cAMP in the regulation of macrophage collagenase production has been further sub-stantiated by the use of agents which influence cAMP metabolism. When βb-adrenergic stimulants, such as isoproterenol or epinephrine, are added at high concentrations (10^{-3} M to 10^{-4} M) to macrophages exposed to endotoxin there is a significant inhibition of both prostaglandins and collagenase whereas at lower concentrations (10^{-6} M to 10^{-7} M) both components are stimulated. A similar profile is observed with dibutyryl cAMP and isobutyl-methyl-xanthine, a phosphodiesterase inhibitor. These findings demonstrate that under conditions in which these drugs should maximally stimulate cAMP both prostaglandins and collagenase production are inhibited. However, when cAMP stimulating agents are added at lower concentrations there is a stimulation of prostaglandins and collagenase. Clearly modulation of cAMP levels within these cells has a direct effect on the production of collagenase. Additionally, while it is known that PG can directly stimulate cAMP these experiments demonstrate that the levels of cAMP can also regulate prostaglandin production through a feed back mechanism.

Our studies on the role of the immune system in bone resorption have focused on the osteopetrosis observed in the toothless (TL) rat. We have shown that the macrophages from these osteopetrotic rats are defective in their ability to regulate lymphocyte proliferation and in their production of prostaglandins. Splenic lymphocytes from these affected animals are hyperproliferative in response to mitogens as compared to lymphocytes from normal littermates. The proliferative difference between these two populations is significantly reduced when the adherent cells are removed from the spleen cells of the normal littermates. These results suggest that the macrophage of the osteopetrotic rat are defective in the production of suppressors of lymphocyte proliferation. Since prostaglandins are known to suppress lymphocyte proliferation, the finding that the macrophages from the osteopetrotic rats produce less PG than those from normal littermates may in part explain the hyperproliferation of the lymphocytes from the affect rats. While the low levels of PG produced by macrophages from the osteopetrotic rats may account for some of the lack of suppression of lymphocyte proliferation there appear to be additional suppressive factors which are missing or produced in low quantities by these cells.

SIGNIFICANCE TO BIOMEDICAL AND DENTAL RESEARCH:

Chronic inflammatory lesions such as periodontal disease and rheumatoid arthritis are associated with the destruction of connective tissue. Our present studies have focused on furthering the understanding of the cellular events leading to the degradation of connective tissue. These studies have indicated that prostaglandins and cyclic adenosine monophosphate have essential roles in the production of collagenase by macrophages. Thus, any way in which the production of prostaglandins and/or changes in cyclic nucleotide levels may be modulated would potentially effect the degradation of connective tissue.

The studies involving the osteopetrotic rat model indicate that there are defects in the immune system which may be related to the pathology of this disorder. Characterization of the exact manner in which these defects contribute to excess bone formation will increase our understanding of the role of lymphocytes and macrophages in bone resorption.

PROPOSED COURSE OF STUDY:

Investigations into the mechanisms of macrophage activation leading to the production of collagenase will continue. Since activation occurs coincident with a change in cell morphology the emphasis will be on experiments dealing with changes in cell membrane which will include determination of lipid composition, changing the lipid composition of the membrane by adding various lipids and determining the role of the cytoskeleton on cell membrane function. The possibility of subpopulations of macrophages, based on size, existing in guinea pig peritoneal exudate which have different capabilities in their production of prostaglandins and collagenase will also be examined. Bone resorption studies utilizing the osteopetrotic toothless rat will concentrate on the apparent defect in the macrophages of these animals. The products secreted *in vitro* by macrophages from osteopetrotic rats and their normal littermates will be compared for their ability to stimulate bone resorption and fibroblast proliferation.

PUBLICATIONS:

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3. Abe, S., Steinmann, B. U., Wahl, L. M. and Martin, G. R. High cell density alters the ratio of type III to I collagen synthesis by fibroblasts. *Nature*. 279: 442-444.

4. Schechter, G. P., Wahl, L. M. and Oppenheim, J. J. Suppressor monocytes in human disease. A Review. Eighth Proceedings of the Recticuloendotholielial Society. In press.

5. Wahl, L. M., Olsen, C. E., Wahl, S. M., McCarthy, J. B., Sandberg, A. L. and Mergenhagen, S. E. Prostaglandin and cAMP regulation of macrophage involvement in connective tissue destruction. New York Acad. Sci. 1979. In press.

6. Wahl, S. M. and Wahl, L. M. Lymphokine modulation of connective tissue metabolism. N. Y. Acad. Sci. 1979. In press.

7. Wahl, L. M., Wahl, S. M. and McCarthy, J. B. Adjuvant activation of macrophage functions. In Regulatory Role of Macrophages in Immunity. Academic Press (Ed. Unanue and Rosenthal) In press.

8. Oppenheim, J. J., Koopman, W. J., Wahl, L. M. and Dougherty, S. F. Prostaglandin E_2 rather than LAF produced by mononuclear cells stimulates increases in murine thymocyte cAMP. Cell. Immunol. 1979. In press.

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10. Moore, R. N., Urbaschek, R., Wahl, L. M., and Mergenhagen, S. E. Prostaglandin regulation of colony stimulating factor production by lipopolysaccharide stimulated murine leukocytes. Infec. Immun. In press.

11. McCarthy, J. B., Wahl, S. M., Rees, J., Olsen, C. E., Sandberg, A. L. and Wahl, L. M. Regulation of macrophage collagenase production by 3'-5' cyclic adenosine monophosphate. In press.

12. Wyler, O. J., Wahl, S. M., Cheever, A. W. and Wahl, L. M. Fibroblast stimulation in vitro by soluble products of isolated schistosomal egg granumolas. In press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 DE 00254-02 LMI

PERIOD COVERED
October 1, 1978 - September 30, 1979

TITLE OF PROJECT (80 characters or less)

Microbial Antigens Associated with Specific Adherence

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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Other:	Kolenbrander, Paul	Senior Staff Fellow	LMI	NIDR
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COOPERATING UNITS (if any) Othmar Gabriel, Georgetown University
Floyd C. McIntire, Albert E. Vatter, and George Revis, University of Colorado Medical Center.

LAB/BRANCH
Laboratory of Microbiology and Immunology

SECTION
Humoral Immunity Section

INSTITUTE AND LOCATION
NIDR, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.00	1.00	1.00

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☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Studies are continuing to examine molecular mechanisms by which species of oral actinomycetes adhere to other plaque bacteria and to mammalian cells. Lectin sites which are inhibited by lactose are associated with the surface fimbriae (i.e. pili) of many human strains of Actinomyces viscosus and A. naeslundii. These sites mediate coaggregation of the actinomycetes with certain oral streptococci and hemagglutination of the actinomycetes with neuraminidase-treated human erythrocytes. Thus, interactions of a lectin-carbohydrate type may contribute significantly to the distribution of certain bacteria within the oral cavity.

PHS-6040
(Rev. 10-76)

OBJECTIVES:

The continuing goals of this project are: (i) to identify and characterize microbial surface antigens which are associated with adherence; (ii) to characterize antibodies against these antigens for their ability to block adherence; and (iii) to examine the biological effects of various microbial products on mammalian cells. The present report focuses on adherence mechanisms displayed by human isolates of *Actinomyces viscosus* and *A. naeslundii* and concerns the interactions of these bacteria with certain oral streptococci and with mammalian cells.

METHODS:

This project utilizes many standard biochemical, immunological, bacteriological, and electron microscopic techniques which have been defined. In addition, the hybridoma technique has been employed to produce monoclonal antibodies against the surface fibrils of *Actinomyces viscosus* T14V. The production of monoclonal antibodies against oral microbial antigens represents a collaborative effort with the Clinical Immunology Section.

MAJOR FINDINGS:

Interactions of Oral Actinomycetes with Streptococci and with Human Erythrocytes (RBC). A survey of coaggregation reactions involving human strains of *A. viscosus* and *A. naeslundii* with isolates of *S. sanguis* and *S. mitis* has revealed a system of specific but varied cell-cell interactions which define two groups of actinomycetes and four groups of streptococci. Results obtained from studying coaggregation between members of each actinomycete and streptococcal group favor the hypothesis that these interactions involve lectin-like sites on one member of a coaggregation pair and carbohydrate receptors on the other member. Virtually all human strains of *A. viscosus* and *A. naeslundii* appear to carry surface lectins which are inhibited more effectively by lactose and β-methyl galactoside than by α-methyl galactoside. Sugars have not been found yet which inhibit those coaggregations in which lectin sites are postulated to be on the streptococci. Like certain lectin-carbohydrate interactions, coaggregation reactions all are Ca^{++} dependent and the requirement for this cation appears to be especially important at values of low pH like those often recorded in plaque.

The mechanism by which human strains of *A. viscosus* and *A. naeslundii* cause hemagglutination of human RBC was examined to help reveal possible mechanisms for adherence of the actinomycetes to oral mucosal surfaces. Adherence of many bacterial strains at 37°C appears to involve two sequential steps: (i) the action of soluble extracellular neuraminidase to expose galactose containing receptors on the RBC, and (ii) multivalent attachment to these receptors by many low affinity lectin sites on the bacterial surface. Thus, galactose-specific lectins, associated with the bacterial surface, may function in the attachment of actinomycetes to plaque streptococci and also to certain mammalian cells.

Studies on the Streptococcal Carbohydrates involved in Coaggregation. A number of galactose-specific plant lectins have been screened for their ability to agglutinate strains of *S. sanguis* and *S. mitis*. Ricinus communis agglutinin-120 (RCA-120) reacted only with those streptococcal strains which gave lactose reversible coaggregation reactions. This correlation suggests that RCA-120 binds the streptococcal receptor involved in lactose-reversible coaggregation.

Studies on the cell surface lectin activity of actinomycete cells. Proteinaceous fibrils (i.e. fimbriae or pili) which cover the surface of actinomycete cells have been isolated from three actinomycete strains. These structures appear to carry lectin binding sites for adherence. Two-dimensional immunoelectrophoresis of each fibril preparation has revealed two antigens. It is not known yet whether each antigen is a different type of fibril and whether each is associated with lectin activity. Various approaches are being employed to answer these questions including the use of monoclonal antibodies against the fibrillar antigens. At present, monoclonal antibodies have been prepared against one of the fibrillar antigens of *A. viscosus* T14V, and this antigen has been found to possess lectin activity.

SIGNIFICANCE TO BIOMEDICAL AND DENTAL RESEARCH:

Specific microbial adherence, though widely recognized as the initial event in many host-parasite relationships, remains poorly understood at the molecular level. The results of this project indicate that surface-associated lectins are present on certain oral bacteria and that these molecules may function in the attachment of microorganisms to each other and to mucosal surfaces. These possibilities provide support for the concept that interactions of a lectin-carbohydrate type represent a significant and general mechanism of microbial adherence. A better understanding of microbial adherence at the molecular level may suggest new and useful approaches for the control of certain bacterial infections.

PROPOSED COURSE:

Studies will continue towards characterizing the lectin binding sites on *A. viscosus* T14V and other actinomycetes. These efforts will focus on the production of monoclonal antibodies which block adherence.

Streptococcal carbohydrates from selected strains will be purified by affinity chromatography on RCA-120 columns. Isolated material will be studied to determine whether it represents the streptococcal receptor in lactose-reversible coaggregation.

Studies to examine certain effects of neuraminidase on immune cells will be undertaken.

PUBLICATIONS:

1. Cisar, J.O. and A.E. Vatter. 1979. Surface fibrils (Fimbriae) of *Actinomyces viscosus* T14V. Infect. Immun. 24:523-531.
2. Cisar, J.O., P.E. Kolenbrander and F.C. McIntire. 1979. Specificity of coaggregation reactions between human oral streptococci and strains of *Actinomyces viscosus* or *Actinomyces naeslundii*. Infect. Immun. 24:742-752.
3. Costello, A.H., J.O. Cisar, P.E. Kolenbrander and O. Gabriel. 1979. Neuraminidase-dependent hemagglutination of human erythrocytes by human strains of *Actinomyces viscosus* and *Actinomyces naeslundii*. Infect. Immun. (In press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 DE 00007-19 LMI

PERIOD COVERED

October 1, 1978 - September 30, 1979

TITLE OF PROJECT (80 characters or less)

Studies on the Regulation of Carbohydrate Metabolism in Oral Microorganisms

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Wittenberger, Charles L.	Research Microbiologist	LMI	NIDR
COPI:	St. Martin, Edward J.	Senior Staff Fellow	LMI	NIDR
OTHER:	Beaman, Alfred J.	Biologist	LMI	NIDR
	Wolf, Anitra C.	Biologist	LMI	NIDR

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Microbiology and Immunology

SECTION

Microbiology Section

INSTITUTE AND LOCATION

NIDR, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.25	1.25	2.00

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The mechanisms by which various pathways of carbohydrate metabolism are regulated in oral bacteria continue to be under investigation. Currently, special emphasis is placed on 1) characterization of the enzyme glucosyltransferase (GTase) produced by Streptococcus salivarius and 2) resolving the means by which the synthesis and secretion of this extracellular enzyme are controlled. The crude enzyme complex has been resolved into two catalytically active components. One (GTase I), produces a water insoluble polymer from sucrose while the other (GTase S) catalyzes the synthesis of a water soluble polymer from sucrose. Mutants have been isolated that are missing the GTase I component. Production of the extracellular GTase complex by S. salivarius is completely inhibited by cerulenin. This antibiotic has no effect on DNA, RNA or protein synthesis, but does severely inhibit cellular incorporation of acetate. It appears that GTase secretion is somehow closely linked to the de novo synthesis of long chain fatty acids.

OBJECTIVES:

It is the continuing general purpose of this project to examine fundamental mechanisms by which the biochemical activities of the microbial cell are regulated and to delineate, where possible, the molecular basis for such regulation. This report is a summary of studies that were oriented specifically toward 1) evaluating the nature of the glucosyltransferase produced by *Streptococcus salivarius* and 2) resolving the mechanism by which this extracellular enzyme is secreted by the cell.

METHODS EMPLOYED:

All are standard techniques routine to the type of studies herein described.

MAJOR FINDINGS:

Characterization of glucosyltransferase produced by Streptococcus salivarius. As part of our continuing investigation of the enzyme glucosyltransferase (GTase) and its role in the colonization of tooth surfaces by certain oral streptococci, we have attempted to characterize the enzyme produced by *S. salivarius*. The crude extracellular GTase has been partially purified and resolved into two catalytically active components by DEAE cellulose chromatography. One component catalyzes the synthesis of a water soluble glucan from sucrose and has been designated GTase(S). The other component produces exclusively a water insoluble polymer from sucrose and has been designated GTase(I). GTase(I) activity is somewhat stimulated by primer dextran, while GTase(S) is almost completely dependent upon primer dextran for activity. The isolated components have different electrophoretic migration rates on polyacrylamide gel columns, suggesting that the proteins are different. In these respects the *S. salivarius* GTase complex closely resembles that produced by *S. mutans* strain 6715.

The fact that the *S. salivarius* enzyme complex could be resolved into two components raised the question of whether these components represented different gene products. As a first step to resolving this question, we have employed a genetic approach. Several mutants of *S. salivarius* have been isolated that are defective in the production of extracellular GTase. One of these mutants (GT-22A) has been analyzed in some detail and found to be missing a catalytically active GTase(I) component. This has been established both by DEAE cellulose chromatography and by polyacrylamide gel electrophoresis. Mutant GT-22A does, however, produce a catalytically active GTase(S) component, which by several criteria, appears to be identical to the parent GTase(S) component. These preliminary results suggest, but do not prove, that GTase(I) and GTase(S) are products of different structural genes. It is noteworthy that GT-22A, unlike the parent organism, is unable to adhere to smooth surfaces in an *in vitro* assay. This indicates a critical role of GTase(I) in the colonization of smooth surfaces by this organism.

Mechanism of glucosyltransferase secretion. Last year we reported the development of a resting cell system for studying GTase secretion by *S. salivarius*. Using this system, we have now examined the possible role of fatty acids in the secretory process.

The antibiotic cerulenin is known to be a potent and specific inhibitor of bacterial β-keto-acyl thioester synthetase. This enzyme catalyzes the condensation of fatty acid-acyl carrier protein with malonyl-acyl carrier protein, which results in fatty acid chain elongation. We have found that cerulenin severely inhibits acetate incorporation by S. salivarius and completely blocks the ability of the organism to produce GTase. In contrast, the antibiotic has little or no effect on the synthesis of DNA, RNA or protein. It appears therefore that cerulenin inhibits GTase production by interfering with long chain fatty acid synthesis. These results further suggest that the inhibitory effect is at the level of enzyme secretion rather than enzyme synthesis.

We have considered that cerulenin might exert its effect by causing an alteration in the fatty acid composition of cell membrane lipids. Such an alteration could affect membrane fluidity and thereby prevent secretion of the enzyme. This was tested by growing the organism in medium containing a concentration of cerulenin that inhibited GTase production. These cells were then harvested, washed free of the antibiotic, and tested for their ability to produce GTase in fresh medium. It was found that cells grown in the presence of cerulenin produced GTase as well as cells grown without the antibiotic. Thus, any membrane lipid alterations that may have occurred during growth of the organism in medium containing cerulenin have no effect on the ability of those cells to subsequently synthesize and secrete the enzyme. Moreover, cerulenin has been found to exert its inhibitory effect more or less immediately after addition to cells producing the enzyme. This too militates against the antibiotic acting to cause extensive alteration of the fatty acid composition of membrane lipids.

Taken together, the available data are more consistent with the hypothesis that cerulenin blocks GTase production by inhibiting some post-translational event involved in its secretion from the cell. This event (or events) appears to require de novo long chain fatty acid synthesis and we are currently exploring the possibility that this may be coupled to a covalent modification of a secretory form of GTase.

SIGNIFICANCE TO BIOMEDICAL AND DENTAL RESEARCH:

These studies on the mechanism of GTase secretion and its regulation in oral microorganisms are undertaken with a broad view toward advancing our state of knowledge concerning the means by which the cell coordinates and controls its diverse biochemical activities. Such information is clearly of broad biological significance. More specifically, however, certain of the microorganisms under investigation have been strongly implicated as etiological agents of oral diseases. It is further anticipated, therefore, that this work will lead to a more comprehensive understanding of how these bacteria successfully colonize and subsist in the complex oral ecosystem. Results from our studies on glucosyltransferase secretion by oral streptococci may also provide insight into possible means of artifically controlling its production.

PROPOSED COURSE:

In general, our studies on exoenzyme secretion by members of the oral microflora will be continued. Special emphasis will be placed on resolving the role of fatty acids in the secretion of the S. salivarius glucosyltransferase. Attempts will also be made to more fully characterize the isolated individual components of the enzyme complex.

PUBLICATIONS:

1. Marucha, P. T., Keyes, P. H., Wittenberger, C. L. and London, J. A rapid method for the identification and enumeration of oral Actinomyces. Infect. Immun. 21: 786-791, 1978.

2. Crow, V. L., and Wittenberger, C. L. Separation and properties of NAD- and NADP-dependent glyceraldehyde-3-phosphate dehydrogenases from Streptococcus mutans. J. Biol. Chem. 254: 1134-1142, 1979.

3. St. Martin, E. J., and Wittenberger, C. L. Characterization of a phosphoenolpyruvate-dependent sucrose phosphotransferase system in Streptococcus mutans. Infect. Immun. 24: 865-868, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 DE 00022-13 LMI B

PERIOD COVERED

October 1, 1978 - September 30, 1979

TITLE OF PROJECT (80 characters or less)

Comparative physiology of lactic acid bacteria and other oral microbes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	London, Jack	Research Microbiologist	LMI	NIDR
Other:	Chace, Nina M.	Chemist	LMI	NIDR
	Celesk, Roger	Guest Worker	LMI	NIDR
	McCabe, Robert	Bio Lab Tech	LMI	NIDR

COOPERATING UNITS (if any)

Paul Keyes, Clinical Microbiology

LAB/BRANCH

Laboratory of Microbiology & Immunology

SECTION

Microbiology Section

INSTITUTE AND LOCATION

NIDR, NIH, Bethesda, MD 20205

TOTAL MANYEARS: 3.50	PROFESSIONAL: 2.75	OTHER: .75

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Xylitol, ribitol and D-arabitol can be utilized by certain strains of Lactobacillus casei and Streptococcus avium via a pathway unique to these microorganisms. The pentitols eventually enter the pathway of pentose metabolism and the ensuing heterofermentation of the sugar alcohols results in the production of lactate, acetate and ethanol. Regulation by glucose is not stringent and several of the enzymes involved in pentitol dissimilation continue to be synthesized while glucose is metabolized.

Gram negative isolates from subgingival plaque deposits have been characterized as Cytophaga sp. These bacteria selectively colonize the root surfaces of teeth, softening and demineralizing the root structure in the process. The initial attachment of the organism to hydroxylapatite appear to be mediated by certain protein and lipid components of the cell's outer membrane.

PHS-6040
(Rev. 10-76)

OBJECTIVES:

A. A study describing the pathway by which the pentitols, xylitol, ribitol and D-arabitol are utilized by certain lactic acid bacteria continues. Currently, the mechanisms involved in the regulation of biosynthesis and catabolite inhibition of the pentitol PEP-phosphotransferase system (PTS) and pentitol phosphate dehydrogenase are under investigation. The following goals are of immediate interest:

1) To determine how the flow of carbon through the pentitol pathway is regulated at both the level of enzyme biosynthesis and enzyme function.
2) Characterize the xylitol, D-arabitol and ribitol catabolizing pathways in Streptococcus avium.
3) Determine the impact of pentitol based sugar substitutes on ecology of oral microflora.

B. Characterization of a group of related Gram negative facultatively aerobic Cytophaga sp. isolated from plaque deposits of patients with chronic periodontitis. The primary objectives of this project are:

1) To characterize this group of bacteria by conventional morphological, biochemical and genetic procedures.
2) Determine method of attachment to root surface of human teeth.
3) Ascertain conditions of growth leading to induction of a functional tricarboxylic acid cycle during aerobic growth and determine regulation of carbon flow through the pathway.

METHODS EMPLOYED:

Conventional immunological biochemical and bacterialogical methods were employed in the studies reported here.

MAJOR FINDINGS:

A. Pentitol dissimilation by lactic acid bacteria. The ability to utilize pentitols is a rare physiological trait in the microbial world and among lactic acid bacteria is restricted, thus far, to two species, i.e., Streptococcus avium and certain strains of Lactobacillus casei. In both organisms, the initial steps in the pathway of pentitol utilization are unique among bacteria capable of dissimilating these five carbon sugar alcohols. A specific PEP-phosphotransferase system and pentitol phosphate dehydrogenase phosphorylate and then oxidize pentitol to pentulose 5-P whereupon the latter compound enters the pentose pathway resulting in a heterofermentation that produces ethanol, acetate and lactate.

Regulation of pentitol metabolism by glucose is not stringent and appears to be effective only at the level of inhibition of pentitol PTS synthesis. And, if preformed pentitol PTS is present in cells growing in a medium containing both glucose and pentitol, the latter will be transported into the cells to provide inducer for the synthesize of pentitol-P dehydrogenase while the cells metabolize and grow at the expense of the hexose.

Attempts to study and characterize the systems regulating the dissimilation of pentitols by transferring the genes coding for the pentitol PTS and dehydrogenase using plasmid pAMβ_1 as a vector or modifying extant transformation procedures were unsuccessful. Neither xylitol or ribitol utilization was expressed in related, recipient strain of bacteria that were initially unable to utilize pentitols. However, pAMβ_1 was shown to be freely transmissible among streptococci, pediococci and lactobacilli.

B. Characterization of Gram negative bacteria from the periodontal pocket and their binding properties. A group of 22 morphologically similar gram negative bacteria have been isolated from the apical area of the periodontal pockets and characterized physiologically and genetically. These thin, flexuous rods are motile by gliding and exhibit limited saccharolytic abilities fermenting only four to six sugars. They grow both aerobically and anaerobically and differ from Capnocytophaga sp. in that they exhibit no CO_2 requirement for growth. This group of bacteria closely resemble facultatively anaerobic members of the genus Cytophaga and are now considered to be members of that genus.

Six of seven strains tested to date have exhibited the ability to adhere to and colonize the tooth root surfaces of human teeth in in vitro experiments. The areas beneath the colonized portions of the root became demineralized and soft similar to the action produced by Streptococcus mutans colonizing teeth. The processes differ in that glucan-like polymers do not appear to be involved in the adhesion of the Gram negative organisms.

Model systems employing spheroidal hydroxylapatite beads (SHA) and sized, powdered root fragments have been devised to study the mechanism of adherence of the Cytophaga sp. to root surfaces. The adsorption of these bacteria to root powder or SHA appears to involve outer membrane protein and lipid components by virtue of the fact that adsorption could be blocked or inhibited by the following treatments. (1) Heating at 70°C for 15 min reduced binding by 50%. (2) Exposure of the cells to proteinases and phospholipase C and D reduced binding by 80%. (3) Binding was blocked by pretreating the SHA or root powder with endotoxin or lipopolysaccharide extracted from the Cytophaga sp. cell walls. (4) Binding was also blocked by pretreating the substrate with outer membrane vesicles isolated and purified from culture supernatants.

PROPOSED COURSE OF RESEARCH:

A. The regulatory mechanisms controlling pentitol metabolism will be elucidated by creating mutants of L. casei and S. avium blocked in either hexose or pentitol utilization and determining the effects of adding potential inducer and/or repressor substrates to growing cultures of the mutants. The pathway of ribitol, D-arabitol and xylitol dissimilation in S. avium will be characterized and the prevalence of pentitol-utilizing bacteria in oral cavity will be determined.

B. Complete characterization of hydroxylapatite binding components in outer membrane of Gram negative bacteria. An attempt will be made to isolate and chemically define components in the cell wall responsible for attachment. The physiological study of enzymatic reactions involved in transition from anaerobic to aerobic states of growth will be continued. The source or reservoir for Cytophaga sp. and establish relationship between oral Cytophaga sp. and soil forms will be determined.

PUBLICATIONS:

1. Marucha, P.T., Keyes, P.H., Wittenberger, C.L., and London, J.: A rapid method for the identification and enumeration of oral Actinomyces. Infect. Immun. 21:786-791, 1978.
2. Gibson, E.M., Chace, N.M., London, S.B., and London, J.: Transfer of plasmid-mediated antibiotic resistance from streptococci to lactobacilli. J. Bacteriol, 137:614-619, 1979.
3. Johnson, J.L., Phelps, C.F., Cummens, C.S., London, J. and Gasser, F.: Taxonomy of the Lactobacillus acidophilus group of organisms. Inter. J. Syst. Bact. In press.
4. Celesk, R.A., McCabe, R.M. and London, J.: Colonization of the cementum surface of teeth by oral gram negative bacteria. Infect. Immun. In press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01-DE-00042-9 LMI B

PERIOD COVERED October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)

Utilization of Carbohydrates by Oral Bacteria

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Bruce M. Chassy	Research Chemist, LMI, NIDR
Other: Porter, E. V.	Chemist, LMI, NIDR
Hull, E.	Biological Lab. aide, LMI, NIDR
Rokaw, E.	Chemist, LMI, NIDR

COOPERATING UNITS (if any)

LAB/BRANCH Laboratory of Microbiology and Immunology, NIDR

SECTION Laboratory of Microbiology

INSTITUTE AND LOCATION NIDR, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 3.50	PROFESSIONAL: 2.00	OTHER: 1.50

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The metabolism of sucrose and lactose has been studied because of these sugars involvement in supporting the formation of dental caries in conjunction with the growth of Streptococcus mutans and Lactobacillus casei. Sucrose can be transported into S. mutans cells by the action of a PEP:PTS activity with the production of intracellular sucrose-6-phosphate (S6P). S6P has been chemically synthesized, and an enzyme catalyzing its hydrolysis to glucose-6-phosphate and fructose has been identified and purified. It is apparently identical with the previously described intracellular invertase. A fructokinase that catalyzes the ATP-dependent phosphorylation of fructose has been characterized as well. In L. casei it has been shown that lactose is transported into the cell by a specific lactose PEP-PTS; the resulting lactose-6-phosphate is cleaved by phospho-β-galactosidase to glucose and galactose-6-phosphate. It has been shown that these first two steps of the metabolism of lactose are plasmid-coded. Several distinct lactose plasmids have been identified and their genetic potential and inter-relatedness is under investigation.

OBJECTIVES:

The oral streptococci and lactobacilli comprise the majority of bacteria found in the oral cavity. These organisms all require fermentable carbohydrate for growth and energy, and they all produce lactic acid. The ability to concentrate and utilize carbohydrates effectively, in an ecosystem where carbohydrates are frequently scarce, has probably been a major selective pressure on these organisms. Some species, such as Streptococcus mutans and to a lesser extent Lactobacillus casei, have been implicated in the formation of carious lesions of the teeth. Fermentable carbohydrates, especially sucrose or lactose, are required for this process.

1) One objective of this study is to understand how sucrose and lactose are dissimilated by various oral streptococci and lactobacilli. It is hoped that such knowledge will contribute to our understanding of both the evolution of these oral microbes and some specific factors which contribute to their pathogenicity.

2) A second major objective has been to determine what role, if any, plasmids have played in the adaptation of oral bacteria to their esocystem. Determination of the genetic potential of the cryptic plasmids found in some species of oral streptococci and lactobacilli is necessary in order to reach conclusions about the adaptive role hypothesized for these plasmids. Since plasmids have been implicated in coding for pathogenic potential in other microbes, that possibility is being examined for certain members of the oral microflora.

This study has demonstrated that lactose fermentation is a plasmid-associated trait in certain strains of L-casei. This specific aspect of the project is directed at elucidating other traits, such as pathogenic potential, associated with these plasmids. The specific genotypic characteristics of the plasmids and their structural and evolutionary inter-relatedness is also under investigation.

MAJOR FINDINGS:

A. Synthesis of sucrose-6-phosphate:

St. Martin and Wittenberger have shown that S. mutans 6715-10 transports sucrose by a specific PEP:PTS with concomitant formation of intracellular sucrose-6-phosphate (S6P). It appeared likely that an enzyme capable of hydrolyzing S6P to glucose-6-phosphate (G6P) and fructose was also present in this strain. Both synthetic and enzymatic methods exist for the synthesis of sucrose-6'-phosphate (S6'P) and the compound has been prepared by an improved enzymatic synthesis in this laboratory. However, S6P is not commercially available and a method for its chemical synthesis has not previously been reported. In this study a chemical means for the synthesis of S6P has been developed. Using cyanoethylphosphate and dicyclohexyl-carbodiimide in pyridine, sucrose was phosphorylated to produce a mixture of isomers comprised of 62% S6P, 24% S6'P and small amounts of other isomers. Homogeneous S6P was obtained by ion exchange chromatography on Dowex-1-chloride employing a linearly decreasing gradient of sodium borate in ammonium chloride. The resulting S6P, representing a 27% yield based on sucrose, was chromatographically and analytically pure. Enzymatic analysis established a minimum content of 97% S6P.

B. Characterization of S6P hydrolase and its identity with intracellular invertase.

Extracts of *S. mutans* 6715-10 (cultured on sucrose) hydrolyzed S6P to G6P and fructose. It was found that *S. mutans* strains E49, BHT, SL1, and LM-7 had similar activity. Cells of these strains cultured on glucose or ructose contained S6P hydrolase activity, although levels were 2 to 5-fold lower than those observed after growth on sucrose. Extracts of *S. mutans* 6715-10 cells were subjected to DEAE-cellulose and Ultragel AcA 54 column chromatography to purify the S6P hydrolase. A 56% recovery of a 71-fold purified enzyme was obtained. Polyacrylamide gel electrophoresis revealed that the preparation was essentially homogeneous. The enzyme has a pH optimum of 7.1 in MES buffer, no metal ion requirement, is quite stable and has a molecular weight of 42,000.

It was observed that the previously described intracellular invertase co-purified with S6P hydrolase. The ratio of S6P hydrolase to invertase activity remained constant throughout the purification. In addition, both activities co-migrated in a single band upon disc gel electrophoeresis. The identity of the two enzymes is further indicated by the observation that sucrose is a competitive inhibitor of S6P hydrolysis with a Ki of 8 mM. Since the Km for S6P hydrolysis is 0.19 mM and the Km for sucrose hydrolysis 90 mM, the enzyme should be considered a S6P hydrolase rather than an invertase. As such it represents a previously uncharacterized enzyme. The action of S6P hydrolase provides G6P for entry into the glycolytic pathway and liberates fructose.

C. Purification and properties of fructokinase from *S. mutans*:

During growth on sucrose, intracellular fructose is produced by the action of S6P hydrolase on S6P. Previous studies in this laboratory had demonstrated the presence of a manno-fructokinase catalyzing the ATP-dependent phosphorylation of fructose or mannose in extracts of *S. mutans*. The enzyme has been highly purified, but instability has prevented the isolation of a completely homogeneous protein. The Km for fructose phosphorylation is 0.63 mM and for mannose 0.37 mM. The molecular weight, determined by gel filtration, is 49,000. The two phosphorylating activities co-purify. Fructose is a competitive inhibitor of mannose phosphorylation and mannose is a competitive inhibitor of fructose phosphorylation. In addition, the two activities co-migrate during disc gel electrophoresis. These results indicated that the same protein catalyzes both activities. The action of this kinase would enable the fructose moiety of sucrose to be assimilated by glycolysis. Moreover, under growth conditions where the phospho-transport system for fructose or mannose is inactive (eg: high substrate concentration or low pH as has been observed for glucose-PTS activity) the enzyme would allow phosphorylation of mannose or fructose that entered the cell via a permease.

D. Purification and properties of glucokinase from S. mutans:

Under conditions of high substrate concentration or low pH the glucose-PTS of S. mutans can be inactivated. A highly specific glucokinase has been isolated from cells of S. mutans during this study. The enzyme would provide an alternative pathway for phospho-rylation of glucose facilitating its entry into the glycolytic pathway. Glucokinase would also serve to phosphorylate any intracellular glucose that accumulates, such as would occur during the fermentation of lactose or maltose. The kinase has been purified to homogeneity by DEAE-cellulose, Ultragel AcA 54 and ATP-affinity gel column chromatography. It has a Km for glucose phosphorylation of 0.61 mM, a molecular weight of 41,000 (SDS gel electrophoresis and gel filtration), and requires ATP and divalent metal ion for activity. It exhibits ordered Bi Bi reaction kinetics typical of several other ATP-dependent glucokinases previously studied.

E. Plasmid-determined β-galactoside metabolism in Lactobacillus casei subsp. casei.

Previous studies in this laboratory had shown that loss of the 23 mdal plasmid, pDRI101, from L. casei DR1002 resulted in loss of the ability to ferment lactose. This same loss of lactose fermentation has been observed with five additional strains of L. casei after treatment with agents known to cause deletion or curing of plasmids. L. casei DR1004 harbors a single 17.5 mdal plasmids which is absent in lactose negative mutants, while L. casei DR1005 loses a 36 mdal lactose plasmid when it loses the ability to ferment lactose. L. casei DR1005 lac variants retain the 4 and 7 mdal cryptic plasmids found in the parent strain. It has been determined that lactose fermenting strains of L. casei subsp. casei have a lactose-PTS for the transport and phospho-rylation of lactose. The lactose-6-phosphate thus formed is cleaved to galactose-6-phosphate and glucose by phospho-β-galactosidase. The galactosyl moiety of lactose, as well as galactose itself, has been shown to be metabolized by the tagatose-6-phosphate pathway rather than the classical Leloir pathway. Loss of these lactose plasmids results in the loss of the ability to ferment all β-galactosides (eg: lactulose, lactobionic acid, methyl-β-galactose, and lactose). Lac isolates are devoid of lactose-PTS activity as well as phospho-β-galactosidase. It is proposed that the structural genes for at least part of the lactose-PTS and for phospho-β-galactosidase reside on the lactose plasmids, while the genes of the tagatose-6-phosphate pathway appear to be chromasomally encoded.

Other traits associated with the lactose plasmids have not been established, however lac$^-$ variants of all three strains differ from their parent strain in resistance or sensitivity to arsenite, arsenate, chromate and cadmium ions. Cell wall analysis has revealed no difference in cell wall composition. No evidence for conjugal functions, proteases, antibiotic resistances or resistance to UV radiation has been found to be associated with the lactose plasmids. Preliminary attempts at restriction enzyme mapping of the plasmid DNA have shown that no obvious homology exists between the three lactose plasmids isolated from three strains of *L. casei*.

SIGNIFICANCE TO BIOMEDICAL RESEARCH

The mechanism of carbohydrate utilization by oral microbes has doubtless been an important selective criterion in their evolutionary development. These studies underscore the diverse and complex adaptation of these organisms to efficiently utilize carbohydrates. Ultimately, such studies should allow us to understand the economy of these organisms and may suggest changes that can be made in the oral environment that would favor shifts in the ability of the ecosystem to support a benign rather than a pathogenic microflora.

Considering the demonstrated role of plasmids in coding for pathogenic potential, confering antibiotic resistance, as well as coding for antigen and bacteriocin production in other bacteria, it would be of great value to know if such relationships exist between plasmids and the oral microflora. In addition, a study of the distribution, function and relatedness of these plasmids may contribute to our understanding of bacterial evolution, specialization, and adaptation in an emerging and changing ecosystem.

PROPOSED COURSE:

1. To characterize fully S6P hydrolase in *S. mutans* 6715-10 and to compare it to S6P hydrolases isolated from other *S. mutans* strains.

2. To conduct a comparison of S6P hydrolase and extracellular invertase from *S. mutans*, as well as to assess their relative physiological roles.

3. To develop a genetic transfer system such as transformation, conjugation or transduction for the analysis of lactose plasmid gene products. Lactobacillus phages being isolated in this laboratory may be exploited in developing a transductional system. As an alternative, this objective may be met by utilizing a cell-free DNA-directed protein synthesis system. Cloning lactose plasmid genes into suitable host organisms is also a possibility.

4. to determine the structural and evolutionary relatedness of the lactose plasmids and to explore other lactose plasmid associated traits. Traits associated with other plasmids found within the genus will be investigated as well.

PUBLICATIONS:

1. Chassy, B. M., E. M. Gibson and A. Giuffrida (1978), Evidence for Plasmid-Associated Lactose Metabolism in _Lactobacillus casei_ subsp. _casei_, _Current Microbiology_ 1, 141-144.

2. Donkersloot, J. A., U. Flatow, E. Gibson and B. M. Chassy (1978). Characterization of Glucosyltransferase-Deficient, Plasmid-Containing Mutants of _Streptococcus mutans_ LM-7. _Infection and Immunity_ 21, 320-327.

3. Chassy, B. M. and E. V. Porter (1979). Initial Characterization of Sucrose-6-Phosphate Hydrolase and its Apparent Identity with Intracellular Invertase. _Biochem. Biophys. Res. 89_: 307-314.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 DE-00043-09 LMI

PERIOD COVERED
October 1, 1978 - September 30, 1979

TITLE OF PROJECT (80 characters or less)
Physiological and Genetic Studies on Pathogenic Oral Microorganisms

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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Microbiology Section

INSTITUTE AND LOCATION
NIDR, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The possibility that an endogenous endo-dextranase contributes to the structural complexity of the adherent and insoluble glucan synthesized from sucrose by oral streptococci is currently under investigation. For this purpose, Streptococcus salivarius mutants that produce either more or less dextranase have been isolated. Whereas the high-dextranase mutant synthesized about the same amount of insoluble glucan and produced adherent deposits like the parent, the low-dextranase mutant produced more soluble glucan and did not form adherent deposits. Two dextranase-deficient mutants of S. mutans 6715-13 have been identified and these also synthesized more soluble glucan. In order to determine whether these phenomena are causally related, the amounts of the glucosyltransferase enzymes producing soluble and insoluble glucan will be assessed. The possible role of plasmids in the physiology of S. mutans is being investigated also. As a result of these studies, two new species of circular DNA have been isolated from strain LM-7. The larger one is a 30Mdal covalently closed molecule, and the smaller one is a 2Mdal circular species, which is not a supercoil and which appears to originate from the previously described 3.6Mdal pAM7 plasmid.

OBJECTIVES:

The ongoing research has two general objectives, which will be discussed sequentially.

1. One focal plane of our attention has been to increase our understanding of the enzymology of insoluble glucan synthesis by oral streptococci. The insoluble glucan synthesized by S. mutans is complex because it is highly branched and contains sequences of both α-(1 → 6)- and α-(1 → 3)-linked glucose residues. At least two functionally distinct glucosyltransferase (GT) activities are present in many strains, GT-I and GT-S, that catalyze the synthesis of an insoluble and a soluble glucan, respectively. GT-I mediates the synthesis of a relatively linear α-(1 → 3) glucan, and GT-S a primarily α-(1 → 6)-linked glucan that is probably branched. Thus, it is evident that the native insoluble glucan is more complex than the product synthesized by GT-I. Several strains of S. mutans secrete a third enzyme, endo-dextranase, and it has been suggested that the action of this enzyme might account for the greater complexity of the native insoluble glucan. This dextranase preferentially hydrolyzes α-(1 → 6)-linked glucans to isomaltose saccharides. If this enzyme acts on the polymer synthesized by GT-S, and if the products are re-incorporated during glucan synthesis, the synthesis of an insoluble glucan similar to the native one can be conceived. This year we have begun to test this hypothesis by studying mutants with altered dextranase activity.

2. The second general objective of our studies has been to characterize the structure and function of plasmids in oral streptococci. So far, the major thrust of our experimental effort has been to critically examine the possibility that a plasmid controls glucan synthesis by S. mutans.

METHODS EMPLOYED:

For most of our studies on glucan biosynthesis, S. salivarius 25975, rather than S. mutans, has been used because it produces much higher levels of GT and because this organism has been employed in several other studies in our laboratory.

Three new methods were used: (1) because the previously described in vivo method to detect dextranase activity was not sensitive enough for S. salivarius, a new method was developed (see below). (2) A staining method was developed which allows partial characterization of the glucan products made in gels by GT-I and GT-S after electrophoretic separation. (3) A new method, recently developed by Don LeBlanc and Linda Lee of our section, has been used to examine S. mutans for plasmid DNA.

Other methods have been described previously.

MAJOR FINDINGS:

Because S. salivarius 25975 did not show dextranase activity on the previously described test agar, a more sensitive test medium was developed for this strain. To prove that this new test agar was, in general, more sensitive, a survey was conducted. It showed that several oral streptococci that had previously been reported as dextranase-negative, did possess activity, because they decolorized the new, blue-dextran containing agar. This medium was then used to detect dextranase mutants after mutagenesis of S. salivarius. Several mutants producing more and one mutant producing less dextranase than the parent were isolated. The high dextranase mutants were similar to the parent with respect to the amount of insoluble glucan synthesized and the formation of adherent, plaque-like, deposits with sucrose. In contrast, the low dextranase mutant synthesized a soluble glucan rather than an insoluble glucan, and did not form plaque-like deposits.

As a result of a survey for dextranase activity among a series of previously isolated mutants with altered glucan synthesis, two other dextranase-deficient mutants were identified. These mutants originate from S. mutans 6715-13 and have been found to synthesize more soluble glucan than their parent, and to be non-cariogenic for smooth enamel surfaces. To see whether the increased synthesis of soluble glucan in the S. mutans mutants was directly due to the dextranase deficiency, GT-I and GT-S activities were assessed after electrophoretic separation. Preliminary evidence from activity stains suggests that the increased synthesis of soluble glucan in these mutants is due to an increased level of GT-S activity, rather than the dextranase deficiency as such. Similar experiments are planned for the S. salivarius mutant.

Our studies on the possible role of plasmids in the physiology of S. mutans are continuing. We have previously shown that, although the ability to form insoluble glucan and to form adherent, plaque-like deposits is lost at relatively high frequency from S. mutans LM-7, this is not accompanied by the loss of the 3.6 megadalton plasmid pAM7. However, the possibility existed that another plasmid that could not be detected by the methods then in use, was controlling this event. Because a new technique to detect plasmids in streptococci had just been worked out, it was applied to S. mutans LM-7. Agarose electrophoresis showed two DNA bands that had not been seen previously. The lower mobility species could be purified by CsCl-ethidium bromide centrifugation, and appears to be a covalently closed circular molecule of about 30 megadalton. However, the smaller species ("X") was lost upon similar centrifugation. Because of this unexpected result, X was studied in more detail. Electron microscopy showed that X had a molecular weight of 2 million. However, despite several searches, a covalently closed molecule of this size was never found, and it was finally decided that the X molecules seen after agarose electrophoresis were in a labile, open circular, configuration. Further work showed that if the adjustment of the cell lysate to pH 12.5, which is part of the plasmid isolation procedure, was omitted, X was not observed. In the next experiment we observed that X was obtained in good yield by subjecting pAM7 to pH 12.5. This shows that pAM-7 contains alkali-sensitive sites. Whether these sites represent modified bases that are alkali-sensitive, or short stretches of RNA, and how the conversion of pAM7 to X takes place, is under investigation.

SIGNIFICANCE TO BIOMEDICAL AND DENTAL RESEARCH:

The studies on the biogenesis of insoluble glucan by oral streptococci are of interest because insoluble glucan formation is associated with the development of plaque on teeth as a result of dietary sucrose consumption, and also with the virulence of *S. mutans* for smooth enamel surfaces. Further characterization of the interrelationships between the glucosyltransferases, dextranase, and insoluble glucan synthesis is also of significance because immunization with glucosyltransferase preparations is being considered as a protective measure against dental caries.

Whereas streptococci have been of clinical significance for a long time, the discovery of plasmids dates from only 1972. Thus, compared with the plasmids from gram-negative organisms, knowledge about streptococcal plasmids is relatively limited. Our studies on the structure and function of *S. mutans* plasmids are designed to advance this knowledge in a general way.

PROPOSED COURSE OF STUDIES:

1. The *S. salivarius* dextranase mutants need to be characterized further. Specifically, the dextranase, GT-I and GT-S activities need to be quantitated. This should answer the question whether the increased synthesis of soluble glucan is solely due to the dextranase deficiency.

2. Because the possibility exists that the dextranase-deficient *S. salivarius* mutant studied so far is, in some unknown way, not representative, other dextranase mutants should be isolated and studied.

3. The two newly discovered DNA species in *S. mutans* LM-7 need to be characterized further. With respect to the larger plasmid, a precise size estimate and a possible function need to be established (does this plasmid control glucan synthesis?). A survey to see if this plasmid is present in other strains of *S. mutans* is contemplated. The finding that the smaller circular molecule ("X") is generated by alkali from pAM7, leads us to probe the nature of the alkali-sensitive sites in pAM7.

PUBLICATIONS:

1. Donkersloot, J. A., Flatow, U., Gibson, E., and Chassy, B. M.: Characterization of glucosyltransferase-deficient, plasmid-containing mutants of *Streptococcus mutans* LM-7. Infect. Immun. 21: 320-327, 1978.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 DE 00096-05 LMI

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)
Microbiological features of Cervico-radicular plaque in healthy and diseased periodontium

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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	McCabe, Robert M.	Bio. Lab. Tech.(Micro)	LMI NIDR

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Bacterial deposits, removed from the subgingival surfaces of teeth having healthy and diseased periodontal tissues, are being assessed by phase-contrast microscopy, fluorescent antibody techniques, and cultural methods. The bacterial complexes associated with disease differ markedly from those associated with health. Phase contrast microscopy reveals different complexes formed by stationary rods, cocci, spirochetes, motile rods, and amoebae. Populations of actinomyces also appear to differ. Some of the motile rods that have been isolated and studied appear to be strains of Cytophaga. Only a few of the highly prevalent spirochital populations have been isolated. Amoebae have been isolated, but maintained with difficulty. As determined by phase contrast microscopy, populations associated with disease can be shifted to those associated with health by antibacterial regimens that include: (1) debridement and decontamination of roots by the clinician, (2) the patient's daily use of certain inorganic salts and hydrogen peroxide to disperse and inactivate root surface bacteria, (3) the occasional administration of tetracycline to suppress the microbiota not reliably controlled by the first two measures. Bleeding, suppuration, ulceration, hyperplasia, tooth mobility, halitosis, and resorption of bone abate as the microbiota associated with disease changes to that associated with health.

OBJECTIVES:

This project is designed to provide answers to the following questions: 1) Does the microflora associated with disease differ from that associated with health? Are the differences merely quantitative (as most dentists believe) or do they also differ qualitatively? Can the use of a phase-contrast microscope in the dental operatory help in the diagnosis and control of periodontal diseases? Can spirochetes, motile rods, amoebae and other bacteria that are prevalent in lesions be isolated and studied? Can target populations be identified and eliminated by appropriate therapy? What happens to teeth after bacterial control has been attained and maintained?

MAJOR FINDINGS:

Samples of bacterial plaque removed from the gingival crevices of persons with no evidence of periodontal inflammation reveal a loosely structured network of filaments colonized by cocci and other small forms. No spirochetes, amoebae, or larger gliding and spinning rods have been detected. Very few white blood cells have been present. Older persons with marginal gingivitis have harboured more dense populations of colonized threads. Spinning rods have been prevalent and in most instances a few spirochetes have been found. A few very long (50 µ+) spiral rods have occasionally been present. Highly turbulent surfaces of spirochetes and larger gliding rods have not been observed. By contrast, samples removed from roots of teeth undergoing autogenous excision reveal highly turbulent surfaces. Static masses of cells are colonized by millions of spirochetes and gliding rods that beat together, often synchronously, setting up wave upon wave of pulsating activity. Numerous amoebae usually glide over the rapidly moving ends of spirochetes and flexing rods. While blood cells, in numbers too numerous to count, assemble in the spaces peripheral to the turbulent surfaces.

A number of solutions can cause almost immediate cessation of all motility and also "coagulation" of the intracellular structures of larger microorganisms e.g.: solutions of sodium bicarbonate, sodium chloride, sodium fluoride, sodium periodate, aluminum chlorate, zinc phenol sulfonate, magensium sulfate, chloramine-T, glycerol, etc. If properly delivered, any of these agents should be beneficial adjuvants for suppressing turbulent populations. All have been used as aids to oral hygiene, some for centuries.

Modified irrigator tips and various applicators are being tried in the clinic and by patients to improve delivery of medicaments to clear, "detoxify", and decontaminate root surfaces and circumradicular spaces. Evaluations of the clinical status of patients, by all conventional parameters of assessment, indicate that periodontal lesions abate or arrest when bacterial populations are reduced to levels the periodontal tissues tolerate, as indicated by low counts of white blood cells in circumradicular spaces.

Teeth malpositioned before being brought under microbiological control, by pressures from high edamatous tissues, have tolerated repositioning without apparent loss of additional tissue.

SIGNIFICANCE TO BIOMEDICAL AND DENTAL RESEARCH:

These studies are designed to explore relationships between the microbial flora and the disease state of the host. It is anticipated that information derived from such studies will ultimately be useful in formulating rational approaches to the diagnosis and treatment of periodontal disease.

PUBLICATIONS:

1. Marucha, P. T., Keyes, P. H., Wittenberger, C. L. and London, J. A rapid method for the identification and enumeration of oral Actinomyces. Infect. Immun. 21: 786-797. 1978.

MITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 DE-00174-04-LMI

PERIOD COVERED

October 1, 1978 - September 30, 1979

TITLE OF PROJECT (80 characters or less)

The Role of Plasmids in the Pathogenesis, Ecology and Taxonomy of the Oral Microflora

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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COPI:	St. Martin, Edward J.	Senior Staff Fellow	LMI	NIDR
OTHER:	Lee, Linda N.	Chemist	LMI	NIDR
	Gibson, Evelyn	Microbiologist	LMI	NIDR

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A rapid, inexpensive and highly reproducible procedure for the detection and isolation of plasmid DNA from virtually any species of Streptococcus was developed and used in all plasmid-associated studies during the past year. The procedure was used to screen 124 tetracycline resistant streptococci, from the oral cavity of periodontal patients, for the presence of plasmid DNA. Plasmids were detected in only 21% of these isolates, suggesting that tetracycline resistance among the oral streptococci is, for the most part, not plasmid-mediated. Using the same procedure, together with standard plasmid curing techniques, data were obtained which suggested that among strains of the group N streptococcus, S. lactis, plasmids play a role in the metabolism of at least six different carbohydrates. The new isolation procedure was also used in the examination of the molecular properties of a deleted antibiotic resistance plasmid in a strain of S. anginosus. The transmissible R factor, pAMβ1, was shown to have lost approximately 50% of its DNA. The resultant molecule was no longer transmissible by conjugation, but still mediated resistance to erythromycin and lincomycin. In addition, the deleted plasmid existed in two unusual molecular configurations.

OBJECTIVES:

The long-range goal of this project remains the elucidation of the role of bacterial plasmids in the pathogenesis, ecology and taxonomic status of the oral microbial flora, with particular emphasis on the streptococci. This report summarizes the results of studies aimed at: 1) developing a rapid procedure for isolating plasmids from streptococci; 2) determining the effects of tetracycline therapy on the oral microbial flora; 3) evaluating the role of plasmids in resistance of the oral streptococci to tetracycline; 4) determining the genetic and molecular properties of a deleted resistance plasmid from a Streptococcus strain; and 5) elucidating the role of plasmids in the metabolism of carbohydrates by strains of S. lactis.

METHODS EMPLOYED:

A new procedure, described below, for isolating plasmids from members of the genus Streptococcus was developed during the past year. Otherwise, all procedures used in these studies were standard microbial or molecular biological techniques, with modifications appropriate for adaptation to streptococci and streptococcal plasmids.

MAJOR FINDINGS:

Development of a Rapid Screening Procedure for the Detection of Plasmids in Streptococci. Standard plasmid isolation techniques have either been too expensive and time-consuming, or totally inadequate, for plasmid screening of large numbers of Streptococcus isolates. By employing critical steps from published enrichment procedures, and incorporating techniques which enhance the lysis of oral streptococci, we have developed a procedure for the isolation and detection of plasmid DNA from virtually all species of streptococci. The procedure is rapid, reproducible and inexpensive, requiring neither radioisotopes nor density gradients for preliminary screening purposes. We have used this technique to screen more than 150 strains of streptococci, including 10 different oral species isolated from clinical sources. Plasmids have been demonstrated in members of all species tested. Several oral isolates previously thought to be devoid of plasmid DNA, following analysis by standard techniques, were shown by our procedure to harbor one or more plasmid species. We have also successfully employed the procedure for preparative purposes, starting with cells from 400 to 800 ml of culture.

Effects of Tetracycline on the Oral Microbial Flora, and the Role of Plasmids in Resistance of the Oral Streptococci to Tetracycline. A 2 and 1/2 year study, designed to determine the effects of short-term tetracycline therapy on the facultative anaerobic subgingival microbial flora of periodontal patients, has been completed. The data indicated that virtually any sample of human subgingival plaque, whether obtained before or after antibiotic therapy, will contain tetracycline resistant bacteria. In the majority of patients studied the resistant population represented a small percentage of the total prior to treatment. However, the proportion of the population resistant to tetracycline increased dramatically (10- to 300-fold) after two weeks of therapy. Withdrawal of the treatment resulted in a significant (3- to 100-fold) decrease in the resistant population within 60 days. The majority of tetracycline resistant isolates obtained at all three sampling times were identified as streptococci. Among 411 tetracycline resistant Streptococcus isolates, 10 species were identified, with S. sanguis (62%) and S. mitis (19%) predominating. There were no significant differences in the distribution of species isolated before, after or following the cessation of tetracycline therapy.

The plasmid enrichment procedure has been used to screen 124 of the tetracycline resistant isolates for the presence of plasmid DNA. Plasmids were detected in only 21% of the strains examined. In two of the plasmid-containing strains it was possible to correlate the tetracycline resistance phenotype with the presence of a specific plasmid species. The results suggest that tetracycline resistance among the oral streptococci is generally not plasmid-mediated. This is particularly significant because tetracycline resistance is probably the most common plasmid-mediated drug resistance found in nature and, historically, has almost universally been plasmid-borne among bacterial isolates from clinical sources.

Unusual Properties of a Deleted Resistance Plasmid. We have previously described the molecular and genetic properties of a transmissible resistance plasmid, pAMβ1 (β), originally isolated from a strain of S. faecalis. The β plasmid has a molecular weight of 17 x 10^6, mediates resistance to erythromycin and lincomycin, and can be transferred between several species of streptococci by conjugation.

S. anginosus strain DR1501 (β) has been used as a universal donor of the β plasmid in conjugation experiments. We have recently isolated a variant of this strain, DR1501/BR1, which is no longer able to donate the β plasmid but has retained the resistance phenotypes.

Plasmid DNA from S. anginosus strain DR1501/BR1 was found to possess the following properties: 1) In agarose gels, DNA bands corresponding to plasmids having molecular weights of 7 x 10^6 (species A) and 10 x 10^6 (species B) were observed, but no 17 M dal band could be seen. 2) A plasmid-less culture of S. anginosus strain DR1501 could be transformed by either species A or species B DNA. All transformants were resistant to erythromycin and lincomycin but could not transfer the resistance traits in conjugation experiments. All transformed clones examined contained both species A and species B plasmid DNA, even though only one of the two species had been used in the transformation. 3) Both species A and B were covalently closed circular (ccc) plasmids, based on dye buoyant density gradient analyses. 4) Analysis of restriction endonuclease digests revealed a molecular weight of 8 x 10^6 for each species, not 7 x 10^6 or 10 x 10^6. The restriction patterns of each species were identical, each missing the same fragments from the original 17 M dal β plasmid. 5) Contour length measurements of relaxed circles of both species were identical, providing a molecular weight estimate of 8 x 10^6. 6) The native ccc molecules had very different configurations, as determined by electron microscopy. Species A appeared as a tightly coiled rosette-like molecule, while species B had a relatively normal ccc configuration.

These results suggest that S. anginosus strain DR1501/DR1 carries a single plasmid from which a 9 M dal segment of the β plasmid has been deleted, and that at least one function required for conjugation has been lost. The remaining 8 M dal segment still possesses replicative functions and antibiotic resistance traits, and can exist in two different, and unusual, ccc configurations.

Plasmid-Mediated Carbohydrate Metabolism in Strains of S. lactis. Among strains of S. lactis the metabolism of glucose, mannose, lactose, galactose, sucrose and fructose is initiated by a phosphoenolpyruvate-dependent phosphotransferase system (PTS). The activities of the sugar-specific components of the PTS for five of these carbohydrates appear to be plasmid-mediated. In one strain of S. lactis, DR1251, the ability to metabolize lactose, galactose and sucrose, and the corresponding PTS activities, were correlated with the presence of specific plasmids. Lactose- and galactose-PTS activities were both dependent on a 32 M dal plasmid in this strain, while loss of sucrose-PTS activity was accompanied by the loss of a 28 M dal plasmid. A second strain of S. lactis, DR1252, was found to become defective in its ability to grow on glucose at a high spontaneous frequency. These glucose-defective isolates were also mannose-defective, in that growth rates on either substrate were considerably slower than in the parent strain, and final growth yields were only half of that obtained with the parent. This dual effect was associated with the loss of a 23 M dal plasmid by strain DR1252. In yet a third strain of S. lactis, DR1253, the ability to grow at the expense of D-xylose, a non-PTS sugar, has tentatively been correlated with the presence of a 3.6 M dal plasmid. All of these results suggest that plasmids play an extremely important role in the metabolism of S. lactis, a Lancefield group N Streptococcus.

SIGNIFICANCE TO BIOMEDICAL RESEARCH:

The contributions of plasmids to the pathogenesis, ecology and taxonomic status of several groups of bacteria have been well-documented. Examples of plasmid-mediated bacterial functions which have had clinical significance are as follows: 1) The widespread occurrence of plasmid-mediated multiple antibiotic resistance continues to frustrate attempts to treat numerous bacterial infections; 2) the enterotoxins responsible for many cases of human and animal diarrhea are now known to be plasmid-coded; 3) the ability of pathogenic coliforms to attach to intestinal epithelial cells, and thus remain in a proper ecological setting for expression of pathogenic traits, is also plasmid-associated; and 4) certain plasmid-mediated metabolic properties, such as lactose utilization in Salmonella, has lead to confusion in the diagnosis of certain disease states. Results from this laboratory, as well as others, have shown that plasmids may play similar roles among the streptococci, at least with respect to antibiotic resistance and metabolic traits.

The overall comparative studies on the molecular biology and genetics of streptococcal plasmids are expected to contribute to a better understanding of these extrachromosomal elements in general. Furthermore, such studies may confirm basic biological principals common to gram-positive and gram-negative bacteria, or perhaps significant differences in the evolutionary patterns of these two major bacterial groups.

PROPOSED COURSE:

Many of our interpretations with regard to plasmid-mediated phenotypic properties among the streptococci have been derived from plasmid curing experiments. During the next year a major effort will be devoted to confirming these results by employing the genetic transfer mechanisms which we have developed over the past three years. In this regard, a protoplast regeneration system, successfully used for the transformation of other gram-positive bacteria, offers some promise of expanding the number of *Streptococcus* species for which transformation may be useful.

An interesting observation made during the screening of the tetracycline resistant clinical isolates was that 18 of 26 strains shown to possess plasmid DNA were classified as either *S. mitis* or *S. sanguis* biotype II. These streptococci are the most difficult to classify because many of the metabolic traits used to differentiate oral streptococci are quite variable among these two species. We plan to investigate the possibility that some of these variable traits are plasmid-mediated. Furthermore, since several of these phenotypic properties are the same carbohydrate catabolic traits thought to be associated with plasmids in *S. lactis*, we will compare the biochemical pathways by which the sugars are metabolized by these *Streptococcus* species.

Finally, other investigators have observed deletions in the β plasmid, and some of these appear to be different from that described by us. By analysis of restriction endonuclease digestion patterns of these deleted molecules we will construct a physical and partial genetic map of the β plasmid. These experiments are also expected to lead to the development of a DNA cloning vehicle for recombinant DNA studies among the streptococci. The availability of recombinant DNA technology would expand our genetic capabilities and facilitate future studies on gene function and regulation in the streptococci.

PUBLICATIONS:

1. LeBlanc, D. J., Crow, V. L., Lee, L. N. and Garon, C. F.: Influence of the lactose plasmid on the metabolism of galactose by *Streptococcus lactis*. J. Bacteriol. 137: 878, 1979.

2. LeBlanc, D. J., Crow, V. L. and Lee, L. N.: Plasmid-mediated carbohydrate catabolic enzymes among strains of *Streptococcus lactis*. In Stuttard, C. and Rozee, K. (ed.). Plasmids and Transposons: Environmental Effects and Maintenance Mechanisms. Academic Press, N.Y. 1979. In press.

3. LeBlanc, D. J., Hawley, R. J., Lee, L. N. and St. Martin, E. J.: "Conjugal" transfer of plasmid DNA among oral streptococci. Proc. Natl. Acad. Sci. U.S.A. 75: 3483, 1978.

4. LeBlanc, D. J., Hawley, R. J., Lee, L. N. and St. Martin, E. J.: Antibiotic-resistance plasmids among the oral streptococci. In Parker, M. T. (ed.). Pathogenic Streptococci. Reedbooks, Surrey, England. p. 286, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF
HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 DE-00208-3 LMI

PERIOD COVERED
October 1, 1978 through September 30, 1979

TITLE OF PROJECT (80 characters or less)
A Genetic Analysis of Metabolic Pathways in Various Members of the Oral Microbial Flora.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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OTHER:	Evelyn M. Gibson	Biologist	LMI NIDR

COOPERATING UNITS (if any)

LAB/BRANCH LMI

SECTION Microbiology Section

INSTITUTE AND LOCATION
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TOTAL MANYEARS: 1.25	PROFESSIONAL: .50	OTHER: .75

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Several metabolic pathways present in Streptococcus mutans have been examined using mutant analysis techniques. Two enzyme systems for ammonia assimilation have been identified: glutamate dehydrogenase and the coupled reactions of glutamine synthetase and glutamate synthase. The primary pathway for sucrose utilization has also been determined. Catabolism of sucrose is initiated by an inducible phosphoenolpyruvate-dependent sucrose phosphotransferase system followed by an intracellular sucrose 6-phosphate hydrolase.

PHS-6040
(Rev. 10-76)

OBJECTIVES:

The general objectives of this project is to use mutant isolation and analysis techniques to examine the function and regulation of several key metabolic reactions present in cariogenic microorganisms. More specifically, we have undertaken the study of ammonia assimilation and sucrose catabolism in S. mutans.

MAJOR FINDINGS:

Ammonia assimilation. We have previously reported that several strains of S. mutans are capable of growth in a chemically defined medium without the addition of an exogenous supply of amino acids. An enzyme, glutamate dehydrogenase, that is capable of fixing free ammonia to form amino acids was detected in these strains. However, examination of glutamate dehydrogenase-negative mutants revealed that they were still capable of growth in the absence of amino acids. Using these mutants, we have identified a second method for ammonia assimilation in S. mutans. This pathway is composed of two enzymes, an ATP-driven glutamine synthetase and a NADH-coupled glutamate synthase, that can function together to fix free ammonia for the biosynthesis of amino acids. The levels of all three enzymes are regulated by the availability of amino acids. This project has been completed.

Sucrose-catabolism. We have identified a phosphoenolpyruvate-dependent sucrose phosphotransferase activity (suc-PTS) in S. mutans. The product of this reaction is sucrose 6-phosphate (suc-6-P). Mutants that are missing either suc-PTS activity or the ability to hydrolyze suc-6-P, were isolated. We have used a mutant that is constitutive for the synthesis of suc-PTS activity but missing suc-6-P hydrolase activity to synthesize substrate amounts of suc-6-P. Using the purified substrate, we have found that suc-6-P hydrolase is a constitutive enzyme with a Km for suc-6-P of 0.3 mM.

SIGNIFICANCE TO BIOMEDICAL AND DENTAL RESEARCH:

The cariogenic potential of oral microorganisms resides in their ability to effectively colonize and proliferate on dental surfaces where they produce acid fermentation products from available carbohydrates. The use of mutant analysis will permit us to determine which of the metabolic reactions possessed by these microbes might be important to their successful colonization of a competitive and changing environment, such as the oral cavity, and permit expression of their pathogenic potential.

PROPOSED COURSE OF STUDIES:

Work on the pathways of sucrose utilization will now focus on enzymes that are capable of hydrolyzing sucrose. In order to examine these reactions, we are using suc-PTS mutants that have regained the ability to metabolize sucrose but retain the original suc-PTS-negative phenotype. Preliminary experiments indicate that these secondary mutants synthesize high levels of an extracellular enzyme that is capable of hydrolyzing sucrose.

PUBLICATIONS:

1. LeBlanc, D. J., Hawley, R. J., Lee, L. N., and St. Martin, E. J. "Conjugal" transfer of plasmid DNA among oral streptococci. Proc. Natl. Acad. Sci. U.S.A., 75: 3484-3487, 1978.

2. LeBlanc, D. J., Hawley, R. J., Lee, L. N. and St. Martin, E. J.. Antibiotic-resistance plasmids among the oral streptococci. p. 286-287. In M. T. Parker (ed). Pathogenic streptococci. Reedbooks Ltd., Surrey, England, 1979.

3. St. Martin, E. J. and C. L. Wittenberger. Characterization of a phosphoenolpyruvate-dependent sucrose phosphotransferase system in Streptococcus mutans. Infect. Immun. 24: 865-868, 1978.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF
HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DE- 00273-01

PERIOD COVERED
October 1, 1978 - September 30, 1979

TITLE OF PROJECT (80 characters or less)

Studies on the Properties of Oral Actinomycetes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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LAB/BRANCH
Laboratory of Microbiology and Immunology

SECTION
Microbiology Section

INSTITUTE AND LOCATION
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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.25	1.00	.25

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The properties of oral actinomycetes which affect the potential of these bacteria to participate in cell-cell interactions with other oral microorganisms are under investigation. Many of the patterns of cell-cell interactions between the oral actinomycetes and oral streptococci have been elucidated. Results from a recent investigation using freshly isolated oral streptococci and actinomycetes not only confirmed earlier results with stock strains from national culture collections but also revealed additional patterns of coaggregation reactions. Currently, an extension of the type of investigation which described the coaggregation patterns between oral actinomycetes and streptococci is being conducted with oral gram-negative isolates. A great deal of specificity between certain Actinomyces israelii isolates and oral Cytophaga isolates has been observed. It is expected that further investigation of bacterial cell-cell interactions will lead to better understanding of the natural mechanisms required to maintain these oral microorganisms in their ecological niche.

PHS-6040
(Rev. 10-76)

OBJECTIVES:

The overall focus of this project is to identify the mechanisms employed by oral bacteria in their interactions with other bacteria and host tissues. This report presents the results of our investigations dealing with:

1) cell-cell interactions (coaggregation) between oral actinomycetes and streptococci.
2) binding properties and specificity of coaggregation between oral *Cytophaga* isolates and actinomycetes.
3) requirement for neuraminidase activity for hemagglutination of human erythrocytes by actinomycete isolates.

METHODS EMPLOYED:

All techniques used are standard methods routine to the type of study conducted.

MAJOR FINDINGS:

Cell-cell interactions (coaggregation) between oral actinomycetes and streptococci. Our initial work provided evidence for delineating four groups of oral streptococci and two groups of actinomycetes based upon the patterns of coaggregation between members of each type of bacteria. Coaggregations occurred frequently when human strains of *Actinomyces viscosus* or *A. naeslundii* were mixed with isolates of *Streptococcus sanguis* or *S. mitis*, but were infrequent or did not occur at all with other oral actinomycetes and streptococci. These experiments were conducted using primarily laboratory stock strains obtained from the Center for Disease Control and the American Type Culture Collection (ATCC). Subsequent studies using more than 300 freshly isolated oral strains both confirm the previous findings for each of the six groups given above and expand the number of groups. One new group of streptococci and two new groups of actinomycetes have been found. In addition, another pattern of coaggregation already noted in the initial studies was again observed with the new oral isolates. This latter pattern was a characteristic of an ATCC stock strain of *Actinomyces odontolyticus*. Since it was the only actinomycete which exhibited that coaggregation pattern in the initial study, it was not well documented. With the discovery of four new isolates with the same coaggregation pattern, it will be possible to define more carefully this important group of oral actinomycetes. It is significant that with such a large number of fresh oral isolates, so few new coaggregation patterns were detected. These results suggest a great deal of specificity is operative in cell-cell interactions between oral bacteria.

Binding properties and specificity of coaggregation between oral Cytophaga isolates and actinomycetes. Initial work with oral streptococcal isolates indicated that none of these coaggregated with *Actinomyces israelii* strains. However, testing of 18 freshly isolated Cytophaga strains revealed that these strains coaggregate only with A. israelii and not significantly with A. viscosus or A. naeslundii. Interactions between members of the latter two species and oral streptococci form the basis of the coaggregation patterns described in the above section. Distinct coaggregation patterns between the Cytophaga isolates and A. israelii strains were observed. Since only four A. israelii strains were immediately avaliable for this study, additional strains have been requested from appropriate sources in order to complete this study. Freshly isolated A. israelii strains will also be tested. It is expected that non-random coaggregation patterns will be observed similar to the results obtained between oral streptococci and A. viscosus and A. naeslundii strains. Already, the specificity of cell-cell interactions between oral bacteria is evident.

1) by the absence of coaggregation between oral streptococci and A. israelii
2) by the interaction between oral Cytophaga isolates and only A. israelii strains
3) by the unique pattern of coaggregation between A. odontolyticus and oral streptococci
4) by the limited number (i.e. specificity) of coaggregation patterns between greater than 300 freshly isolated actinomycetes and streptococci

The binding properties of these Cytophaga isolates and A. israelii with respect to binding to root powder and spheroidal hydroxyapatite are currently under investigation in collaboration with Dr. Roger Celesk and Dr. Jack London of this section. It appears that precoating either of the above solid substrates with Cytophaga cells decreases the binding efficiency of A. israelii as compared to binding of A. israelii to uncoated substrate. This result confirms the existence of cell-cell interaction already indicated by coaggregation experiments between these two groups of bacteria. It also points toward the potential for demonstrating a competition between these two groups of bacteria for solid substrate sites.

Requirement for neuraminidase activity for hemagglutination of human erythrocytes by actinomycete isolates. In addition to binding to other bacteria, oral actinomycetes are able to hemagglutinate human erythrocytes. In collaboration with Dr. John Cisar of the Humoral Immunology Section, we have found that hemagglutination occurs after a period of incubation at 37°C unless the erythrocytes have been pretreated with neuraminidase. In the latter case hemagglutination occurs immediately. Nearly all of the actinomycete strains tested (25 of 26 tested) produce neuraminidase. Part of the activity is cell-bound and the remainder is in a soluble extracellular form. Only the soluble form is effective in pretreatment of erythrocytes for hemagglutination with actinomycete cells, and an absolute requirement for neuraminidase activity for hemagglutination has been demonstrated. The nature of the binding of actinomycete cells to erythrocytes may be similar or identical to that observed between certain actinomycetes and streptococci since it is reversible by lactose. The relationship between the two types of cell-cell interactions is not clear at this time but may be an indication that a wide range of cell types, especially those found in the oral cavity, may participate in these interactions.

SIGNIFICANCE TO BIOMEDICAL AND DENTAL RESEARCH:

These studies provide evidence regarding the prevalence of highly specific cell-cell interactions observed between different species of oral bacteria and between oral bacteria and mammalian cells. It is likely that these interactions contribute significantly to the distribution of these microorganisms within the oral cavity. The actinomycetes are thought to play a role in the etiology of root surface caries and periodontal disease. It is hoped that by investigating the properties of oral actinomycetes, it will be possible to understand the molecular basis for the cell-cell interactions and cell-solid support adherance involving this important group of oral bacteria.

PROPOSED COURSE:

Studies focusing on coaggregation patterns of freshly isolated oral bacteria will be continued in an effort to identify the extent of this phenomenon within the oral cavity. This will include further studies with gram-positive bacteria and will expand the study to include selected gram-negative organisms. Studies on the oral actinomycetes will be central throughout these investigations. Their ability to produce extracellular enzymes such as neuraminidase, collagenase, lipases, etc. will be examined. An attempt will be made to relate these capabilities to their capacity to be maintained in their ecological niche, the periodontal pocket.

PUBLICATIONS:

1. Cisar, J.O., Kolenbrander, P.E. and McIntire, F.C.: Specificity of coaggregation reactions between Actinomyces viscosus or Actinomyces naeslundii. Infect. Immun. 24:742-752, 1979.
2. Costello, A.H., Cisar, J.O., Kolenbrander, P.E., and Gabriel, O.: Neuraminidase-dependent hemagglutination of human erythrocytes by human strains of Actinomyces viscosus and Actinomyces naeslundii Infect. Immun. In press. 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 DE 00034-11 LMI

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Mechanisms of Histamine Release

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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LAB/BRANCH
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SECTION
Clinical Immunology Section

INSTITUTE AND LOCATION
NIDR, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 5 1/2	PROFESSIONAL: 4 1/2	OTHER: 1

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Histamine release from mast cells and blood basophils is being studied as one of the immunological mechanisms involved in inflammation. Among the histamine releasing agents employed are IgE antibody, environmental antigens, and complement factors (C5a and C3a) derived from human serum. The relationships between IgE crosslinking, basophil desensitization and histamine release were analysed by kinetic studies. Release of inflammatory mediators from cultured mastocytoma cells is being compared with mechanisms of release from mast cells and basophils.

I. Project Description

A. Mechanisms of *in vitro* desensitization of human basophils for IgE-mediated histamine release.

Basophils from atopic individuals can be desensitized by incubation with antigen under non-releasing conditions. Cells from human subjects were desensitized *in vitro* with two different environmental antigens and then challenged with the same antigens and anti-IgE. The rate of cell desensitization varied with the antigen concentration. With concentrations of ragweed antigen in the ascending portion of the histamine release curve, desensitization was slower than with supra-optimal amounts of antigen. The reduction of histamine release after desensitization was proportional to the time of incubation. The degree of desensitization at 1 to 37°C was proportional to the incubation temperature. The leukocytes of most subjects tested showed non-specific desensitization to two or more antigens. Desensitization of the cells of a few other subjects showed only a one-way desensitization. Only one individual was found that showed specific desensitization toward two antigens, *i.e.* cells were desensitized specifically to both antigens. The results indicate that *in vitro* desensitization of human basophils is predominantly non-specific and this specificity is not dependent on antigen concentration nor on incubation temperature.

B. Mathematical models for analysis of receptor crosslinking and histamine release in basophils

A mathematical model for relating the number of antigen-induced IgE clusters on the basophil plasma membrane to the amount of histamine released was developed. The method is valid at low doses of antigen where, it is shown, the number of bound antigens and the number of IgE clusters are linearly proportional to the total concentration of antigen. Crosslinking of receptors on some portion of the surface is considered to be a necessary condition for transmission of a signal that leads to degranulation. No assumption is made about whether the degranulation thus stimulated is local or global. Analysis of the low-dose portion of the dose-response curve, however, indicates the following: 1) Degranulation is local, and the maximum number of local centers per cell ("compartments") is small ($\lesssim 10$). 2) The number of IgE clusters required to initiate such local release is small but variable and depends upon the cell source and antigen valence. The results varied between three and eight for Con A, AgE, and antiIgE. 3) These numbers represent thresholds for release; i.e., to a good approximation some minimum number of antigens in excess of one must be multiply bound before *any* histamine can be released. This suggests a high degree of cooperativity in the biochemical sequelae to clustering. 4) Only a small fraction of clusters are transmitting a release signal. This suggests, and is consistent with the idea, that clusters are interacting with some other membrane molecule and that only those that are bound to the other molecule are biologically active. 5) With respect to transmitting a signal for histamine release the number of independent clusters appears to be a more fundamental quantity than the total number of cross-linked receptors; i.e., there is little difference in activity as the size of a cluster increases due to increase in ligand valence.

Analysis was performed of doseresponse and desensitization of rabbit basophils by a variety of antigens. Unlike the response of human basophils, the histamine release curves rarely rise and fall in a symmetric fashion, but more often either rise to a plateau, peak, and then fall to a non-zero plateau, or exhibit bimodality. In those cases in which the curves peak, preincubation with antigen in the absence of Ca^{++} has a smaller effect at the high and low concentration portions of the curve than it does toward the center, where the inhibitory effect is maximum.

The results can be explained by a simple model in which: 1) The signals for release and desensitization both increase as the concentration of receptor cross-links increases and 2) the signals reach a plateau (i.e., they have a maximum value) when the number of receptor cross-links becomes sufficiently large. By changing the parameters of the model, the balance between desensitization and release changes, and all the observed patterns can be reproduced. In particular, if both signals saturate (reach their maximum value) at a ligand concentration below that of maximum cross-linking, the histamine release curve will plateua. If the release signal saturates but the inhibitory signal continues to increase as lgand concentration increases toward its optimum value, the histamine release curve will be bimodal.

Many of the parameters can be identified with those that affect receptor clustering. These include the affinity of IgE for the antigen the number of IgE molecules per basophil, IgE hinge flexibility, membrane fluidity, and antigen concentration and valence. This identification permits a variety of additional predictions. One such prediction is that the difference between a dose-response curve that plateaus and one that is biphasic and unimodal reflects easier desensitization of cells responsible for the latter curve and/or that have a smaller number of cross-links. Both predictions are readily testable experimentally.

Basophils from rabbits sensitized to penicillin release histamine when stimulated with bi- or multivalent penicillin derivatives but not by the monovalent hapten. Monomer inhibits release by either bi- or multivalent antigens. Kinetic studies revealed histamine release curves which were sigmoidal, having delays in the onset of release that varied with ligand concentration. The results were interpreted in terms of a model in which crosslinked receptors are converted to an active, exhaustable intermediate which facilitates an increase in cytoplasmaic Ca^{2+}, but which decays spontaneously into an inactive product. The dose dependence of desensitization is also biphasic and is most pronounced at an antigen concentration which ordinarily stimulates maximal histamine release. The implication is that IgE cross-linking by antigen initiates signals for both degranulation and desensitization of basophils.

C. Efficacy and Specificity of immunotherapy with laboratory animal allergen extracts.

Patients allergic to labroatory animals were studied for their blocking antibody response after immunotherapy with animal allergen extracts. Blocking antibodies were measured by serum inhibition of allergen induced histamine release from washed leukocytes. Blocking antibody titers of treated subjects were significantly higher than those of untreated controls. They correlated with the cumulative allergen dose given during immunotherapy as well as with the final weekly dose. The antibody response of treated patients was allergen-specific. Within two years after immunotherapy was discontinued there was a decrease of blocking antibody titer to levels of untreated patients.

D. Antigenic structure of albumins.

Rat serum albumin (RSA) and bovine serum albumin (BSA) are proteins with significance as allergens for humans sensitive to animals. In order to elucidate the allergenic structure of both proteins, histamine release experiments were done with several peptide fragments prepared by limited peptic and tryptic digestion of the molecules. Screening individuals sensitive to rats revealed that 25% gave histamine release with RSA. The allergenic activity of two peptic fragments were weakly reactive when compared to the whole RSA molecule. Non-releasing fragments could be shown to be of haptenic character by inhibition experiments. In rabbits immunized with BSA, as well as human subjects sensitive to BSA, the peptide fragments of the albumin molecule had little or no antigenic activity. Studies in rabbits indicated the unique nature of these antigenic sites on albumin.

E. Hypersensitivity to food antigens.

Leukocytes from subjects exhibiting oral, gastrointestinal and other manifestations of food allergy were tested *in vitro* for histamine release by common food antigens including cereals, corn, cottonseed, coffee, egg, fish, shellfish, meats, milk, strawberry and tomatoes. The results indicate that there frequently is no correlation between a clinical history of hypersensitivity to food and the antigen-induced in vitro histamine release from human leukocytes. The intriguing observation was also made that a sizeable proportion of subjects without symptoms of food hypersensitivity show IgE-mediated histamine release with the food allergens. This suggests that humans may possess a control mechanism to prevent an allergic response to ingested food antigens.

F. Histamine release by cultured mastocytoma cells.

Mastocytomas induced in mice by Abelson murine leukemia virus were adapted to in vitro cell culture. The mastocytoma cells possess granules containing histamine and serotonin and are useful for studies of the mechanisms of histamine secretion. Four mastocytomas were obtained from four different mouse strains: Balb/C, CXBG, CXBI and CB-ABM-4. The histamine content, IgE and IgG_1-mediated histamine releasing ability of the various cell lines has been studied. Work is in progress toward defining other phenotypic characteristics of these mouse mastocytomas.

The rat basophilic leukemia (RBL) cell line originally obtained by Eccleston is unresponsive to IgE-mediated histamine release. However, it has been possible to obtain variants of the RBL lines which behave like normal animal cells in this property. The releaser and non-releaser clones thus obtained have a chromosome number ranging from 65-75. Both possess the same IgE receptor and their IgE receptor association constants are similar. However, IgE dissociates slower from the non-releaser cell lines. Such RBL clones obtained from the parental tumor cell constitute a homogeneous population of cells that can be utilized to study the complex phenomenon of histamine release.

G. Preparation of hybridomas.

IgE-producing hybrid lines were obtained after hybridizing mouse myeloma (NS-1) and spleen lymphocytes from BALD/C mice parasitized with Nippostrongylus brasiliensis. The antibody producing hybrid cultures were successfully transferred to mice and the ascites fluid obtained from the tumor-bearing animals contained large quantities of IgE. This monoclonal IgE and subsequently a homologous anti-IgE will allow studies of the interaction of IgE with its receptors on mast cells. Monoclonal antibodies were also obtained against rat Fc receptors; this will allow us to study the role of such receptors on mast cells, and to compare the IgE and IgG receptors on these cells.

H. Studies of inhibitors and enhancers of histamine release using the RBL cell line.

The effect of various compounds on the IgE-mediated, antigen-induced histamine release from RBL-1 HR+ (2H3 subline)- was investigated. The cells were sensitized with IgE, preincubated with various pharmacologic agents and challenged with antigen. The metabolic inhibitors - antimycin A, 2,4 dinitrophenol, sodium azide and potassium cyanide inhibited histamine release especially in the absence of glucose while 2-deoxy glucose had minimal or no effect. Agents which react with microtubules had variable effects; cytochalasin B and D enhanced antigen-induced release, whereas cytochalasin A had a biphasic effect: low concentrations $< 10^{-6}$ M enchanced and higher levels inhibited release (10^{-5}M). Vinblastine and colchicine inhibited histamine release whereas D_2O showed mild enhancement. Compounds known to increase intracellular cAMP levels inhibited release only at high concentrations (e.g. 50% inhibitory levels with dibutyryl cAMP, aminophylline and isoproterenol $>5 \times 10^{-4}$M). Cholera toxin caused enhancement at 100 μg/ml. Other compounds which had no effect on histamine release included dibutyryl cGMP, cAMP and carbachol. Release was inhibited by the protease inhibitors TPCK or TLCK and by hetrazan. In general, the release mechanism from RBL-1 HR+ cells appears to have differences from that of normal mast cells.

I. Histamine release by C5a and C3a anaphylatoxins of human serum.

The activation of human serum complement by incubation with microbial cell walls, including dental plaque, generates anaphylatoxin (C5a) which releases histamine from human basophils. These cells also possess receptors for C3a, another anaphylatoxic peptide derived from human serum. On a molar basis, C5a is approximately 1000 times more active in releasing histamine than C3a. The specificity of the receptors for purified C5a and C3a were studied by cell desensitization experiments. It was found that leukocytes desensitized with human C5a retained their sensitivity to release by purified human C3a. Smiliarly, cells desensitized with the chemotactic peptide fMet-Leu-Phe retained full sensitivity to histamine release by both C5a and C3a. The results support the concept that separate, specific receptors for C5a and C3a are present on human basophils.

II. Significance to Biomedical Research and the Program of NIDR

The purpose of the project is to study the immunologic release of mediators such as histamine which represent the effector mechanisms by which IgE, allergens, or microbial cells may interact with leukocytes or serum to cause inflammation. Desensitization mechanisms and pharmacologic agents are studied in vitro for their potential use in control of inflammatory responses.

III. Proposed Course

Experiments will pursue further pathways and regulatory mechanisms of immunologically-induced histamine release.

IV. Publications.

1. Siraganian, R.P. and Harzard, KA.: Mechanisms of mouse mast cell activation and inactivation for IgE-mediated histamine release. J. Immunol. 122:1719-1725, 1979.

2. DeLisi, C. and Siraganian, R.P.: Receptor cross-linking and histamine release. I. The quantitative dependence of basophil degranulation on the number of receptor doublets. J. Immunol. 122:2286-2292, 1979.

3. De Lisi, C. and Siraganian, R.P.: Receptor cross-linking and histamine release. II. Interpretation and analysis of anamolous dose response patterns. J. Immunol. 122:2293-2299, 1979.

4. Siraganian, R.P. and Metzger, H.: Evidence that the "Mouse Mastocytoma" cell line (MCT-1) is of rat origin, J. Immunol. 121:2584, 1978.

5. Siraganian, R.P. and Sandberg, A.L.: Characterization of mouse allergens. J. Allergy & Clin. Immunol. 63: 435, 1979.

6. Siraganian, R.P., Baer, H., Hochstein, H.D. and May, J.C.: Allergenic and biological activity of commercial preparations of house dust extract. J. Allergy and Clin. Immunol. (In press).

7. Hernandez-Asensio, M., Hooks, J.J., Ida, S., Siraganian, R.P. and Notkins, A.L.: Interferon-induced enhancement of IgE-mediated histamine release from human basophils requires RNA synthesis. J. of Immunol. 122:1601-1603, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 DE 00085-06 LMI

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Studies in Sjögren's Syndrome

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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	Pavlidis, Nicholas	Fogarty Fellow	NIDR LMI

COOPERATING UNITS (if any)

Dermatology Branch, NCI	Arthritis and Rheumatism Branch, NIAMDD
Immunology Branch, NCI	Lab. Clin. Invest., NIAID

LAB/BRANCH
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SECTION
Clinical Immunology Section

INSTITUTE AND LOCATION
NIDR, NIH, Bethesda, Maryland 20205

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☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this project is to determine the etiology of the autoimmune disease Sjögren's Syndrome. Particularly we studied the basis of clinical, serologic and genetic similarities and differences between Sjögren's syndrome alone and Sjögren's syndrome associated with other autoimmune disease; the presence and physiochemical properties of immune complexes in these patients, the ability of the Fc receptor of their RES to clear immune complexes, and the immunoregulation of primary Sjögren's syndrome.

PHS-6040
(Rev. 10-76)

Project Description

I. Objectives

To determine the pathophysiology and etiology of Sjögren's syndrome.

II. Methods

1) Clinical studies

a. Study of the clinical manifestations of Sjögren's syndrome in the presence or absence of rheumatoid arthritis. This study was conducted through retrospective analysis of the medical records of 43 patients.

b. Study of the relationship of systemic lupus erythematous (SLE) to Sjögren's syndrome. This study was done in 24 consecutive patients with SLE who were evaluated for clinical, laboratory and histologic findings of Sjögren's syndrome. Each patient answered a questionnaire and underwent a complete eye examination, parotid flow rate, parotid scintigraphy and biopsy of the labial salivary glands.

2) Laboratory studies

a. HLA typing - Lymphocytes from 22 patients with Sjögren's syndrome alone and 11 patients with Sjögren's syndrome and rheumatoid arthritis were typed for HLA-A, -B, -C, -DRW and Ia-like B-lymphocyte antigens. The study was done in collaboration with Dr. Dean Mann.

b. Serologic studies - Sera from 24 patients with SLE, who were tested for clinical and histologic findings of Sjögren's syndrome, 17 patients with Sjögren's syndrome alone and 12 patients with Sjögren's syndrome in the presence of rheumatoid arthritis were tested for rheumatoid factor by the bentonite flocculation test, antibodies to native DNA by ammonium sulfate precipitation test and antibodies to cellular antigens SS-A, SS-B and RANA by immunodiffusion.

c. Circulating immune complexes and the FC receptor function of RES in Sjögren's syndrome.

These studies were done in collaboration with Dr. M. Frank's laboratory. The presence of circulating immune complexes were tested in the serum of 54 patients with Sjögren's syndrome by the ^{125}I-C1q binding assay and the Raji cell radioimmunoassay. Furthermore, the composition of immune complexes was examined. The Fc receptor function of the RES was investigated in 19 patients with Sjögren's syndrome by measuring the rate of elimination of injected chromium labeled IgG-sensitized autologous erythrocytes.

d. Immunoregulation in Sjögren's syndrome - These studies were done in collaboration with Dr. Fauci's laboratory. Peripheral T and B lymphocytes were enumerated by standard techniques. The immunoregulatory T-cell subpopulations were identified on the basis of the presence of Fc receptors for IgG or IgM immunoglobulins. The B-lymphocyte spontaneous activation was defined according to the ability of the peripheral B-lymphocytes to produce plaques to the hapten TNP. In addition the number of circulating intracytoplasmic Ig containing B-cells were enumerated. The B-cell function was evaluated on the basis of their response to polyclonal activator pockweed mitogen. The suppressor function of the T-cells was assessed by the their ability after concanavalin-A stimulation to suppress pockweed mitogen driven cultures.

III. Major Findings

Relationship of Sjögren's syndrome alone to Sjögren's syndrome associated with other autoimmune diseases.

Sjögren's syndrome occurs as an entity alone or in association with other autoimmune diseases, primarily rheumatoid arthritis or systemic lupus erythematosus.

Our retrospective clinical study on the clinical manifestations of Sjögren's syndrome in the absence and presence of rheumatoid arthritis revealed that the two entities have quite distinct clinical features. Patients with Sjögren's syndrome alone had a significantly greater frequency of recurrent parotid gland enlargement, Raynaud's phenomenon, purpura, lymphadenopathy, myositis and renal involvement despite the fact that the salivary histopathology was identical in both groups of patients.

Study on the incidence of autoantibodies to cellular antigens SS-A, SS-B and RANA revealed that patients with Sjögren's syndrome alone have a high incidence of autoantibodies directed to SS-A and SS-B antigens and not to RNA. In contrast partients with rheumatoid arthritis and Sjögren's syndrome produce antibodies to RANA and not to SS-A and SS-B antigens. Thus patients with Sjögren's syndrome alone in addition to clinical differences with patients with Sjögren's syndrome and rheumatoid arthritis possess a serologic dichotomy. To investigate if this dichotomy occurs on the grounds of genetic differences we studied the association of these groups with certain HLA antigens. We found that patients with Sjögren's syndrome alone is highly associated with HLA-B8, HLA-DRW3, Ia-172, Ia-350 and Ia-715 while Sjögren's syndrome in the presence of rheumatoid arthritis is associated with HLA-DRW4 Ia-172 and Ia-350. Thus these two groups of patients have common genetic markers (Ia-172 and Ia-350) and different ones HLA-88, HLA-DRW3, HLA-DRW4 and Ia-715. The lymphocytic infiltrate of the minor salivary glands may depend on the degree associated with Ia-172 and Ia-350 while the gene(s) associated with HLA-B8, HLA-DRW3 and Ia-715 may fulfill a role in patients with Sjögren's syndrome alone similar to that played by rheumatoid arthritis and its associated antigen HLA-DRW4 in patients with Sjögren's syndrome and rheumatoid arthritis. These observations have prompted us to evaluate the characteristics of Sjögren's syndrome in patients with systemic lupus erythematosus. We evaluated 24 patients with systemic lupus erythematosus for clinical (keratoconjunctivitis sicca, xerostomia), histologic, and serologic evidence of Sjögren's syndrome. Clinical abnormalities suggestive of Sjögren's syndrome in patients with systemic lupus erythematosus were identified by parotid scan (58%), questionnaire (54%), labial biopsy (50%), Schirmer's test (33%) and parotid flow rate (13%). The most striking finding, however, was that patient with systemic lupus erythematosus and abnormal labial biopsy had antibodies to SS-A cellular antigen and to immunoglobulins similar to those seen in patients with Sjögren's syndrome alone. Thus in three autoimmune diseases associated with focal lymphocytic infiltrate of the salivary glands distinct antibody responses to different cellular antigens can be demonstrated. We interpret these findings to support the hypothesis that Sjögren's syndrome alone and Sjögren's syndrome in systemic lupus erythematosus are closely related and each differs from Sjögren's associated with rheumatoid arthritis.

Circulating immune complexes and function of the Fc receptor of the RES in Sjögren's syndrome

We have described previously that three patients with Sjögren's syndrome developed immune complex glomerulonephritis. This prompted us to study if immune ceomplexes circulate in patients with Sjögren's syndrome and their physiochemical properties. The sera of 54 patients with Sjögren's syndrome were tested for immune complexes with the ^{125}I-Clq binding assay and the Raji radioimmunoassay. Eighty-five percent of these sera were positive by either assay for immune complexes. This was true of all clinical subcategories and regardless of whether or not typical signs and symptoms of immune complex mediated diseases were present. The two assays showed concordance of 90% in the detection of circulating immune complexes. The amount of immune complexes as detected by the Rajii cell assay was not correlated with the titer of rheumatoid factor, but a positive association was found between the Clq binding activity and the titer of rheumatoid factor. To eliminate the possibility that rheumatoid factor may comprise the major part of the circulating immune complexes in patients with Sjögren's syndrome, sera were pretreated with 2-mercaptoethanol to dissociate the pentameric IgM rheumatoid factor or with solid phase protein

A to absorb IgG and were then analyzed for Clq binding activity and rheumatoid factor. Pretreatment of the sera with 2-mercaptoethanol resulted in complete elimination of rheumatoid factor activity but only slight reduction in Clq binding activity. Furthermore, all the Clq binding activity was removed from Sjögren's syndrome sera by absorption with solid phase protein A. These findings indicate that high percentages of patients with Sjögren's syndrome have circulating immune complexes containing IgG antibody. The fact that the vast majority of Sjögren's syndrome's patients have evidence of circulating immune complexes is interesting especially since most lack the overt clinical signs considered indicative of immune complex disease. It may be that the Clq binding assay and the Raji cell assay are detecting a variety of immune complexes only some of which are capable of mediating severe vascular or glomerular tissue damage, whereas other may be capable of producing the low grade vasculitis seen in Sjögren's patients.

To determine the functional status of the RES Fc receptors in these patients we studied the rate of clearance from the circulation of chromium labeled IgG-sensitized autologous erythrocytes in 19 patients with Sjögren's syndrome. It was found that abnormal clearance was noted particularly in patients with extraglandular disease. In contrast, there was no correlation between the rate of clearance of IgG-sensitized erythrocytes and the serum immune complexes as measured by the Clq binding assay. The striking correlation between clearance rates and disease manifestations suggests that decreased clearance of immune complexes by defective RES Fc receptors may contribute to disease pathogenesis.

Immunoregulation in Sjögren's syndrome

Fifteen patients with Sjögren's syndrome (sicca syndrome) with either glandular or extraglandular involvement, but without other connective tissue diseases, were studied with regard to immunoregulatory T cell subpopulations, B cell function, and suppressor cell capabilities. Patients with isolated glandular disease as well as patients with extraglandular disease had normal absolute numbers of total lymphocytes, T cells, and B cells. However, six of eight patients with extraglandular disease and only two of seven patients with glandular disease had decreased relative proportions of T_G cells which was explained by a factor that blocked the expression of the IgG Fc receptor on T_G cells. This blockage was reversible since the factor could be removed by trypsinizing the T cells before T_G determination. Serum from patients with low proportions of T_G cells, but not serum from patients with normal proportions of T_G Cells, blocked the expression of the IgG Fc receptor on normal T cells. The serum factor upon fractionation over Biogel A 1.5 columns was found in a diffusely distributed pattern including IgG but excluding IgM.

Neither patients with glandular nor extraglandular disease manifested increased numbers of in vivo activated circulating lymphocytes as determined by spontaneous anti-TNP PFC. However, patients with glandular disease had reduced numbers of PWM-induced anti-SRBC PFC ($p < 0.01$) as compared to normals and patients with glandular disease. Of note was the fact that despite the modulation of T_G subpopulation by the serum factor in patients with extraglandular disease, these patients manifested normal Con A-generated suppressor cells of PWM-induced PFC responses in allogeneic co-cultures. This was unlike the suppressor cell defect previously described in this system with SLE patients. The discrepancy was attributed to the fact that the T_G defect was reversible as well as the fact that Con A-generated suppressor cells are not limited to the T_G subsets. Thus, these studies have demonstrated reversible abnormalities in T_G cells with extraglandular Sjögren's syndrome which are not associated with suppressor cell defects. The discrepancy between these findings and the immunoregulatory defects demonstrated in SLE may explain the difference in severity of the autoimmune expression in these diseases.

IV. Publications

1. Kassan SS, Thomas TL, Moutsopoulos HM, Hoover R, Kimberly RP, Budman DP, Costa J, Decker JL and Chused TM: Increased risk of lymphoma in sicca syndrome. Ann. Inter. Med 89:888-892, 1978.

2. Gottdiener JS, Moutsopoulos HM, Decker JL: Echocardiographic identification of chardiac abnormality in scleroderma and related disorders. Amer. J. Med. 66:391-398, 1979.

3. Moutsopoulos HM, Webber BL, Vlagopoulos TP, Chused TM, Decker JL: Differences in the clinical manifestations of sicca syndrome in the presence and absence of rheumatoid arthritis. 66:733-736, 1979.

4. Chused TM, Moutsopoulos HM, Sharrow S, Hansen CL: Evidence of a primary B-lymphocyte abnormaility in NZB mice. In genetic control of autoimmune disease. NR Rose, PE Bigazzi, NL Warner, (eds) Elsevier, Amsterdam 1979, pp 177-191.

5. Hooks JJ, Moutsopoulos HM, Geis SA, Stahl NI, Decker JL, Notkins AL: Immune interferon in the circulation of patients with autoimmune disease. New Engl. J. Med. 301:5-8, 1979.

6. Taurog JD, Moutsopoulos HM, Rosenberg YJ, Chused TM, Steinberg AD: CBA/N x-linked B-cell defect prevents NZB-B-cell hyperactivity in F_1 mice. J. Exp. Med. 150:31-43, 1979.

7. Simon BF, Moutsopoulos HM: Primary amyloidosis resembling sicca syndrome. Arthritis and Rheum. In press 1979.

8. Moutsopoulos HM, Mann DL, Johnson AH, Chused TM: Genetic differences between primary and secondary sicca syndrome. New Engl. J. Med. In press 1979.

9. Lawley TJ, Moutsopoulos HM, Katz SI, Theofilopoulos AN, Chused TM, Frank MM: Circulating immune complexes in Sjögren's syndrome. J. Immun. In press 1979.

10. Hamburger MI, Moutsopoulos HM, Lawley TJ, Frank MM: A defect in reticuloendothelial system FC receptor function in Sjögren's syndrome. Ann. Inter. Med. In press 1979.

11. Moutsopoulos HM, Klippel JH, Pavlidis N, Wolf RO, Sweet JB, Steinberg AD, Chu FC, Tarpley TM: Correlative histologic and serologic findings of sicca syndrome in patients with systemic lupus erythematous. Arthr. Rheum. In press 1979.

12. Reinertsen JL, Shaefer ES, Brewer HB, Moutsopoulos HM: Sicca-like syndrome in type-V hyperlipoproteinemia. Arthr. Rheum. In press 1979.

13. Moutsopoulos HM, Fauci AS: Immunoregulation in Sjögren's syndrome. Influence of serum factors on T-cell subpopulations. J. Clin. Invest. In press 1979.

14. Moutsopoulos HM (moderator): Sjögren's syndrome. Current issues. NIH conference. Ann Inter. Med. In press Dec. 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 DE 00114-05 LMI

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Control Mechanisms of IgE Production

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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COPI:	Fox, Philip C.	Clinical Associate	NIDR LMI
OTHER:	Fischler, Cynthia	Medical Technician Micro.	NIDR LMI

COOPERATING UNITS (if any)

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1 1/2	1/2	1

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The control mechanisms of IgE production are under study utilizing a mouse model system. Attempts are being made to purify murine IgE and develop a radio-immunoassay capable of quantitating the small amounts of IgE present in mouse serum (estimated to be less than 1.0 μg/ml): for this purpose an IgE-hydridoma has been produced. Mice exposed to nebulized antigens are incapable of an IgE response when later immunized with the same antigen. The mechanism of this phenomenon is under investigation.

PHS-6040
(Rev. 10-76)

PROJECT DESCRIPTION

I. Objective:

The aim of this project is to better define the control mechanisms involved in the production of IgE. A model system in the mouse is utilized for this purpose. Murine IgE is being purified for further studies utilizing sensitive radio-immunoassays, fluorescent techniques and tissue culture studies.

II. Method and Description:

A. Purification of Mouse IgE. The purification of mouse IgE would make available a useful reagent for studies of the cellular control of IgE production. This purification is being attempted through the use of mouse ascitic fluid and classical techniques (ammonium sulfate precipitation between 40 and 50%, DEAE separation followed by gel filtration). These steps result in ∿ 100 x purification. Removal of other contaminants is achieved through the use of solid phase immunoabsorbants prepared with antibodies to the contaminants. The method seems to be capable of purifying IgE even though it is present in serum in very small amounts. The recent development of hybridizing normal spleen cells with myeloma cells to produce "hydridomas" producing monoclonal antibodies has been utilized to obtain an IgE producing cell line. The IgE has been purified from the tissue culture fluid. The hybridoma cells have been injected into mice and ascitic fluid rich in IgE has been obtained.

A rabbit antibody to mouse IgE has been prepared and it will neutralize the mouse 72 hr PCA activity at a concentration of 2 μg of protein. Further use is being made of histamine release from mouse mast cells to quantitate and follow the purification of both the anti-IgE and antibodies being prepared to impurities in the IgE.

B. IgE Suppression Due to Exposure to Nebulized Antigen. Mice exposed to different nebulized antigens are incapable of an IgE response when later immunized with the same antigen. The suppression is antigenically specific and is observed even after a short exposure of mice to a nebulized antigen. The suppression is observed only for the IgE class of antibodies, the mice mount a normal IgG_1 response. At present, experiments are underway to determine whether the suppression is at the level of T or B cells and whether it is effective in turning off an ongoing immune response. Experiments suggest that this suppression is due to a factor(s) in serum and may be transfered with serum from nebulized antigen exposed mice.

III. Significance:

Mediator release from mast cells and basophils is an important amplification system in immunological injury. IgE - antigen interaction is the specific trigger for the release of histamine. The present studies are an attempt to better understand the mechanisms which control the induction and synthesis of IgE. The mouse model is ideal for this type of study because of the extensive previous studies on cellular interactions in this animal and the availability of multiple inbred strains.

IV. Preposed Course:

1. Studies will determine the characteristic of the phenomenon of IgE suppression induced by exposure to nebulized antigen. The cellular basis of this suppression will be investigated.

2. Purified mouse IgE will be used in studies of the immunobiology of the IgE system; e.g. studies on the interaction of IgE with mast cell.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 DE 00205-3 LMI

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Investigation of Cellular Immunological Mechanisms in Periodontal Disease

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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	Oppenheim, J.	Medical Officer	NIDR LMI
	Hook, William A.	Research Microbiologist	NIDR LMI
	Siraganian, Reuben	Chief, Clinical Immunology	NIDR LMI
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COOPERATING UNITS (if any)

None

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1 3/4	1 3/4	0

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☒ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A group of patients with periodontosis is being studied to identify cellular immunologic deficiencies. They are being compared to patients with juvenile and adult periodontitis and individuals without periodontal pathology. A a familial pattern of this disease is being investigated.

PROJECT DESCRIPTION

I. Objective:

The study group consists of individuals with periodontal disease, including a sub-group of young people with periodontosis, a severe localized juvenile form of periodontitis. The aim of this project is four-fold:

1) To determine the immunologic status of patients with periodontal disease.

2) To identifty specific immune reactions in patients with different forms of periodontitis.

3) To attempt to differentiate periodontosis from other periodontal diseases (periodontitis and juvenile periodontitis) in a systematic manner.

4) To investigate familial patterns of periodontosis.

II. Method and Description:

All patients are screened for underlying medical conditions or vitamin deficiencies. A full oral exam is carried out which includes dental radiographs and a complete periodontal evaluation. Immune function is studied utilizing several assay systems including lymphocyte transformation, lymphokine production, monocyte and neutrophil chemotaxis and histamine release. Gingival fluid is analysed for cellular components. Dental plaque is evaluated for its' antigenic potential in these patients. Immune responses to pure cultures of other oral microorganisms are studied.

Groups studied include individuals with adult and juvenile periodontitis of varying severity and normals. Chemotactic activity was studied in response to: 1) partially purified C5a derived from zymosan activated serum; 2) crude bacterial factor derivd from E. Coli; and 3) fMet-Leu-Phe. Results of the chemotactic assay demonstrate that patient and control neutrophil function was the same. Monocyte chemotactic activity was noted to be deficient in some cases studied. There was a great deal of variability noted in responses of normal and patient cells on repeated testing. Several factors were identifed which appeared to effect chemotactic responsiveness. These included: antibiotics, smoking, aspirin, antihistamines and other drugs. There was a difference noted in chemotactic response of monocytes to self or foreign dental plaque. Plaque itself was not a chemoattractant. Lymphocyte transformation and histamine release studies showed a correlation between cell responsiveness and degree of gingival inflammation, with inflammation serving as a better predictor of response than severity of bone loss. HL-A frequencies were unremarkable.

Recent studies, in collaboration with Dr. Zvi Metzger, have examined the role of macrophages in the lympho-proliferative responses of periodontal disease patients. Differing degrees of reactivity to oral and plaque micro-organisms, as well as certain ubiquitous antigens, have been correlated with disease severity. Macrophage depletion has altered the level of reactivity in the patients studied.

III. Significance:

Periodontosis is a unique form of periodontal disease in several respects; 1) it occurs early in life (from puberty to age 30); 2) it is extremely localized in its expression in the mouth, and 3) it is characterized by extensive bone loss without gingival inflammation. It has been reported that patients may have a systemic cellular defect. The absence or presence of this defect is important to document as it may contribute to an understanding of the mechanisms of bone loss in periodontal disease. The local expression of such a systemic defect would provide a valuable model for the study of cellular immune interactions.

IV. Proposed Course.

1) Additional patients are being recruited to extend these studies.

2) Any differences noted in our study group will be intensively investigated for expression in other than the oral environment.

3) If a defect can be defined, efforts will be made to treat these patients by overcoming this defect with local or systemic therapy.

4) Several new assays will be utilized to better differentiate subtle differences between control and study groups. Additional chemotactic techniques are being investigated to resolve the question of a chemotactic defect in periodontosis patients.

V. Publications

1. Olsson Wennström AO, Wennström JL, Mergenhagen SE and Siraganian RP: The mechanism of basophil histamine release in patients with periodontal disease. Clin. exp. Immunol. 33:166173, 1978.

ANNUAL REPORT OF THE LABORATORY OF ORAL MEDICINE NATIONAL INSTITUTE OF DENTAL RESEARCH

The Laboratory of Oral Medicine is concerned with the etiology and pathogenesis of diseases of the soft tissue of the oral cavity with emphasis on: 1) viral infections such as herpes simplex virus; 2) aphthous ulcers and other dermatologic disorders; 3) exocrine and endocrine diseases with special attention to diseases of the salivary glands and pancreas (e.g., diabetes mellitus); and 4) malignant and premalignant lesions of the oral cavity. The program is disease oriented and highly interdisciplinary.

Over the last year, in-depth studies have continued on the projects discussed in the 1977-78 annual report. In addition, one new project was initiated and one project was terminated. The new project is concerned with receptors on the plasma membrane of cells. There are two aspects of this project; the first deals with viral receptors and how they influence susceptibility to infection; the second involves alterations of other types of plasma membrane receptors and how they are changed by viral infections. The terminated project dealt with "targeting" or "homing" of biologically active mediators (e.g., chemotactic factor) to specific sites (e.g., tumors, viral lesions) by antibody molecules. This project was determinated because of the departure of the two principal investigators.

The progress in the Laboratory since the last annual report has been excellent. We have at last achieved a critical mass of investigators with training and experience in virology, immunology, pathology, electron microscopy, cell biology and molecular biology. Many of our studies on the etiology and pathogenesis of disease require an interdisciplinary approach and over the last year, this approach has proved to be particularly fruitful.

Emphasis on human diseases and clinical material from patients continues; this is particularly true for the projects on interferon and diabetes. This coming fall, a clinical investigator from Scotland with both a D.D.S. and M.D. will be joining our staff as a Visiting Associate. In addition, a senior investigator from the University of California, whose clinical expertise is infectious diseases, will spend his sabbatical year with our group. The only recruitment at the present time is to find a full-time, clinically-oriented investigator to replace Dr. Howell Archard who will be retiring this fall.

The various service components of the Laboratory continue to function well and this is particularly the case with the Tissue Culture Unit. A major effort has been made over the last year to reorganize and modernize our deep freezer system for storage of viruses and cell cultures. Efforts also are being made to renovate and modernize some of our space so that it can be more effectively utilized. In the Office, a Lexitron typewriter was purchased to more efficiently prepare manuscripts.

The Laboratory continues to add new techniques and methods to its program. This year we have set up methods for: 1) making antibody-producing hybridomas, 2) measuring antiviral antibody by microtitration, 3) detecting viral and other antigens by the peroxidase-anti-peroxidase method, 4) measuring the number of viral receptors on the surface of cells, 5) quantitating insulin receptors, 6) enriching for herpes simplex viral DNA sequences, and 7) restricting HSV DNA by endonucleases.

A number of new collaborative projects have been initiated with investigators from NIAID, NIAMDD, and NCI. In addition, case material is being sent to us from at least one-half dozen universities throughout the country.

Since the last annual report, good progress has been made in several areas. The significance and implications of some of our findings are briefly summarized below:

1. Previous studies showed that type II or immune interferon could be produced by peripheral blood leukocytes in vitro on an immune-specific basis. This suggested that it also might be produced in vivo in various autoimmune disorders. Over the last year, we found immune interferon in the serum of a high percentage of patients with systemic lupus erythematosus, rheumatoid arthritis, scleroderma and Sjogren's syndrome. Subdivision of the patients with lupus erythematosus into active and inactive disease showed that approximately 71% of those with active disease had interferon in their circulation as compared to only 21% of those with inactive disease. Moreover, serial serum samples obtained over many months showed a good correlation between interferon titers and disease activity. Thus, it is possible that the production of interferon may on one hand contribute to immunological aberrations in autoimmune diseases, while on the other hand protect the already compromised host from viral infections. The possibility that type II interferon may be found in the circulation of patients with a variety of other immunological disorders is now under active investigation.

2. New findings have been made in the area of virus-induced diabetes. Since the last annual report, members of the Laboratory have succeeded in isolating a virus from a child who died of diabetic ketoacidosis and showed that this virus, a variant of Coxsackievirus B4, could produce diabetes in certain inbred strains of mice. A variety of factors including a rise of antibody to the virus during the patient's illness and the presence of viral antigens in the patient's brain argue against the virus being a laboratory contaminant. Although cases of this type are a rarity, it supports the hypothesis that some cases of juvenile-onset diabetes mellitus may be triggered by viruses and backs up the years of experimental work in animal models.

A second line of evidence supporting the hypothesis that viruses can sometimes produce diabetes comes from histopathologic evaluation of pancreas from patients dying of overwhelming viral infections. These studies showed that viruses can infect and damage pancreatic beta cells.

New studies with EMC virus have shown that the virus pool is not homogenous but contains both diabetogenic and non-diabetogenic variants. Moreover, the non-diabetogenic variant can block the capacity of the diabetogenic variant to produce diabetes. This has important implications for our understanding of how viruses produce beta cell damage and diabetes.

3. Studies have continued on the role of immunity in the host's defense against herpes simplex virus infection. Last year, we showed that both the humoral and cellular components of the host's immune system were needed to eliminate the acute phase of the ganglionic infection. We now have evidence that once latency is established, it can be maintained in the absence of antiviral antibody. This observation provides the basis for a variety of new experiments on the mechanism of latency.

The model system for studying HSV reactivation described last year has been extended. LOM investigators now find that it is possible to reactivate HSV in the trigeminal ganglion of at least a small percentage of animals by direct trauma to the lips. This system may be analogous to reactivation in humans.

By use of molecular hybridization techniques, evidence has been obtained that HSV is not only latent in the trigeminal ganglion, but also in the brain stem and cerebral hemispheres. If this observation can be extended to humans, it is possible that HSV may play a role in chronic and degenerative diseases of the central nervous system.

4. Last year it was shown that fmet-leu-phe (a powerful chemotactic agent) could be coupled to antibody against HSV and that the resulting molecule retained its capacity to bind to viruses and also was chemotactic. This year we showed that epidermal growth factor (a powerful stimulant of cell division) also could be coupled to antibody against HSV and that the resulting molecule retained its antibody binding capacity and was mitogenic. This indicates that a variety of biologically active mediators can be coupled to antibody molecules.

A detailed description of these and other projects follows.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 DE 00080-06 LOM

PERIOD COVERED
October 1, 1978 - September 30, 1979

N01 DE 92420-01

TITLE OF PROJECT (80 characters or less)

Diseases of the Salivary Glands and Pancreas: Virus-Induced Diabetes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
7.30	4.55	2.75

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Diabetogenic variants of encephalomyocarditis virus, Coxsackievirus B, and reovirus have been studied in vitro in beta cell cultures and in vivo in animal model systems. One of these variants, a Coxsackievirus B4, was isolated from the pancreas of a child who died with acute juvenile-onset diabetes (JOD). The role of the immunologic system in JOD also is being studied.

PHS-604D
(Rev. 10-76)

DISEASES OF THE SALIVARY GLANDS AND PANCREAS: VIRUS-INDUCED DIABETES

Background

Juvenile-onset diabetes (JOD) occurs at an early age and is characterized by an acute onset, appearance of islet cell antibody (ICA), an infiltration of inflammatory cells in and around pancreatic islets (insulitis), and a reduction in the number of insulin-containing beta cells. Because of the abrupt onset and development of insulitis, an infectious etiology has long been suggested for JOD. Seroepidemiologic studies and numerous case reports have shown that a temporal relationship exists between the onset of certain virus infections and the subsequent development of diabetes. JOD has been reported to follow infection by members of the Coxsackievirus B group (particularly B4) within days, mumps virus within weeks to years, and congenital rubella by a number of years. This line of evidence suggests that a number of viruses either acting alone (Coxsackieviruses) or interacting with host factors such as the immune system (mumps and rubella) may cause JOD in susceptible individuals.

Some viruses have the capacity to infect and damage human pancreatic islet cells in vitro. We have previously shown that Coxsackieviruses B3 and B4 and mumps virus can infect and degranulate human beta cells in culture. Although these studies did not prove that viruses can actually infect and destroy beta cells in vivo to produce JOD, they did provide a sound basis for the idea that viruses play an active role in JOD.

The best evidence that viruses cause diabetes comes from experiments with an animal model system. Infection of certain strains of mice with the M-variant of encephalomyocarditis (EMC) virus results in the development of a diabetes-like syndrome which closely resembles JOD. This variant of EMC infects and destroys murine beta cells and produces hypoinsulinemia and hyperglycemia. Only certain inbred strains of mice develop EMC virus-induced diabetes which appears to be genetically controlled at a single recessive locus.

Murine beta cells grown in culture are susceptible to the M-variant of EMC virus, Coxsackievirus B3, Coxsackievirus B4, and reovirus type 3. Although the M-variant of EMC is a naturally occurring diabetogenic virus, the Coxsackieviruses and reoviruses mainly cause an acinar cell pancreatitis. However, after passage of reovirus type 3 and Coxsackievirus B4 through murine beta cells grown in culture, it was possible to obtain variants of these viruses which, when inoculated into mice, infected the beta cells and produced hypoinsulinemia, hyperglycemia and/or abnormal glucose tolerance tests. The diabetogenic effect of reovirus type 3 could be assessed most accurately by performing glucose tolerance tests whereas the diabetogenic effect of EMC and Coxsackievirus B4 was best assessed by determining levels of nonfasting glucose and insulin at varying times after virus infection.

Objectives

The objectives of this research project are to determine 1) which viruses cause diabetes, and 2) the sequence of events that occur between the time of infection and the onset of clinical disease.

Major Findings

STUDIES IN ANIMALS

EMC: Isolation of a diabetogenic clone: We have previously shown that the diabetogenic capacity of the M-variant of EMC virus is markedly diminished after passage in mouse kidney cell cultures. One passage through animals, however, restored its diabetogenic capacity. We suggested that the stock virus pool was made up of two populations of virus; one that had a tropism for beta cells and was diabetogenic, and the other that did not have a tropism for beta cells and was not diabetogenic. Growth of virus in the kidney cell cultures might have favored replication of the nondiabetogenic strain. In vivo, both beta-tropic and nonbeta-tropic strains would have an opportunity to replicate as true stable variants.

To obtain a genetically uniform stock of diabetogenic virus, the M-variant of EMC virus was grown in secondary mouse embryo cells (SME) and purified by density gradient centrifugation. The purified virus was passaged five times in cultures enriched in murine beta cells. The virus was then passaged in mice and harvested from hearts. By serial plaque purification of the passaged virus, two distinct stable clones were obtained. When these clones were tested in SJL/J mice, one of them (D-clone) produced hyperglycemia in up to 100% of the animals. In contrast, the other isolate (B-clone) did not induce diabetes. Virus titers were checked in the islets and heart from mice at different times after infection. At two days after infection, the amount of infectious virus recovered from the islets of D-clone infected mice was at least 10-fold higher than recovered from B-clone infected mice. Similarly, the D-clone replicated in the heart to a greater extent than the B-clone. Moreover, microscopic examination of sections of pancreas from D-clone infected mice revealed that more than 80% of the islets developed insulitis (lymphocytic infiltration) and necrosis of beta cells. In contrast, sections of pancreas from B-clone infected mice showed that about 90% of the islets appeared normal. Using immunofluorescence, about 97% of the islets from D-clone infected mice (2 days after infection) contained specific viral antigens, while only 13% of the islets from B-clone infected mice contained viral antigens. Further studies showed a marked decrease in the insulin content of the pancreas from D-clone infected mice, beginning at 24 hours after infection. In contrast, infection of mice with B-clone resulted in no significant change in insulin content of the pancreas. These studies clearly showed that the newly isolated diabetogenic clone (D-clone) that is genetically stable, differed from the non-diabetogenic clone (B-clone) in its capacity to replicate in the intact animal. We are now trying to determine if the differences in replication of these clones in vivo are due to the responses they ellicit in the host (e.g., antibody, interferon) or are due to molecular changes in the virus that affect its function at the cellular level.

Quantitative evaluations of islet cell populations in EMC-infected mice. In order to determine the quantitative variations occurring in the different endocrine cells of the islets during virus-induced diabetes, SWR/J male mice were inoculated with the M-variant of EMC virus and sacrificed at different time intervals following infection. Insulin-containing B-cells, glucagon-containing A-cells, pancreatic polypeptide-containing PP-cells, and somatostatin-containing D-cells were identified by immunofluorescence using specific antibodies against the respective hormones. A marked reduction of the volume of insulin-containing B cells (up to one-tenth of control values) was observed at all stages studied in the hyperglycemic mice. This was accompanied by the inversion of the normal ratio between B- and non B-cells. The volume of the latter cell types was also modified at different time points after infection: glucagon-cells were augmented 14 days after infection; PP-cells were decreased 2-3 days and 21 days after infection; somatostatin-cells decreased to one-fourth of control values in hyperglycemic animals 21 days after infection. The latter results suggest that non B-cells are also involved in islet reaction to virus infection.

Reovirus. Reovirus is widely distributed in the human population, and more than 80% of the people have antibody to reovirus. Its role in human disease, however, is not clear. Last year we showed that reovirus type 3, passaged in mouse beta cells, produced a diabetes mellitus-like syndrome in mice by infecting and damaging primarily the insulin-producing beta cells.

This year a diabetogenic variant of reovirus type 1 was obtained by passage of prototype reovirus type 1 (Lang strain) in mouse pancreatic beta cell cultures. Newborn mice were infected with 10^6 PFU and at different times after infection, the concentrations of insulin and glucagon in the pancreas and in the blood were determined. The amount of insulin and glucagon was decreased by approximately 50% at day 5. Immunoreactive insulin and glucagon remained depressed over the next 10 days and then began to return toward normal levels.

A number of infected animals showed a distinctly abnormal response to a glucose load. Abnormal glucose tolerance tests were first detected at 10 days after infection and were still apparent in most animals 40 and 60 days later.

Morphological change was observed by light microscopy and electron microscopy. Within 7 days after infection, a mild insulitis was usually seen. By electron microscopy, reovirus particles (70 mm in diameter) in different stages of morphogenesis were observed in insulin-containing beta cells, as well as glucagon-containing alpha cells and somatostatin-containing D cells. These developing virus particles were frequently associated with microtubules in large cytoplasmic virus factories.

Frozen sections from the pancreas of infected mice were stained with fluorescein-labeled anti-reovirus type 1 and rhodamine-labeled antiinsulin antibody. Reovirus antigens were found in both beta cells and noninsulin-containing islet cells. By the peroxidase-anti-peroxidase method using anti-glucagon antibody, more than 50% of alpha cells were degranulated at days 14 and 21. It is concluded that a variant of widely disseminated human virus, reovirus type 1, can infect the alpha, beta and D cells of mouse pancreas. The damage of these cells alters the hosts' capacity to respond normally to a glucose load.

Microculture system. Pancreatic islet cells have been grown in monolayer cultures, but this technique has not been widely used because of the difficulty in obtaining large numbers of cells and maintaining them in a functional state. Monolayer cultures are particularly suitable for studying immune phenomena. For these reasons, we have developed a functional microculture system that would require relatively few cells and make available large numbers of cultures.

Preliminary studies reported last year have been confirmed and extended to include new data. The cultures were prepared in 96 well tissue culture trays from islet cells isolated from enzymatically disrupted newborn rat pancreas. The cultures could be maintained in a functional state for at least 2 weeks as defined by their responsiveness to known modulators of insulin secretion. Glucose, for example, enhanced insulin release, as did arginine leucine and isoleucine. These agents are known stimulators of insulin secretion in vivo. As few as 1×10^3 cells per well gave results that were reproducible within ± 10%.

Experiments are currently underway to study the biological significance of islet cell antibody using this system. The effects of ICA on islet cell function as well as its cytotoxic properties are being investigated.

STUDIES IN HUMANS

Efforts are being made to determine which viruses may be involved in human JOD. We are following three approaches. The first approach is to isolate viruses from the pancreas of patients dying with acute JOD, and then characterize the virus in tissue culture and in animal model systems. The second approach is to determine which viruses have a tropism (in vitro) for human beta cells in culture. The third approach is to look at the islets of patients dying with fatal viremia and determine if these viruses produce cytopathology in islets.

Coxsackievirus B4: A healthy ten-year-old male developed a flu-like illness and within three days after onset of symptoms, the child was admitted to the hospital with diabetic ketoacidosis and died seven days later. Microscopic examination of sections of the pancreas revealed lymphocytic infiltration of the islets of Langerhans and necrosis of insulin-producing beta cells. Small pieces of the pancreas were removed at autopsy and stored at -70°C. Monolayers of secondary mouse embryo cells, Vero cells and human embryo kidney cells were inoculated with a 10% homogenate of the frozen pancreas. Six days later, 10 to 20% of the monolayers showed one or more focal areas of cytopathology. The cells and media were harvested, frozen and thawed three times, and then passaged twice in secondary mouse embryo cells. The material obtained after the second passage produced large plaques on secondary mouse embryo cell monolayers.

To characterize the virus, sera obtained from mice that had been infected with the human isolate neutralized the human isolate and also the mouse-adapted diabetogenic variant of Coxsackievirus B4. These results indicate that the human isolate is closely related to a diabetogenic variant originally derived from Coxsackievirus B4. To further characterize the virus, monolayers of secondary mouse embryo cells grown on coverslips were infected with the human isolate and then stained with FITC-labeled antiserum to the mouse-adapted variant of Coxsackievirus B4, Coxsackievirus B3, and EMC virus. Cells stained with FITC-labeled antiserum to the mouse-adapted variant of Coxsackievirus B4 showed brilliant fluorescence in the cytoplasm, while uninfected control cells did not fluoresce. Monolayers infected with the human isolate and then stained with FITC-labeled antiserum to Coxsackievirus B3 or EMC virus failed to fluoresce.

Evidence that the virus was not a laboratory contaminant came from showing: 1) a rise in antibody titer to Coxsackievirus B4 during hospitalization, and 2) viral antigens in brain tissue obtained at autopsy. Serum obtained on the second hospital day failed to neutralize the human isolate at a dilution of 1:4, while serum obtained six days later neutralized the isolate at a dilution of 1:32. In contrast, neither serum obtained on the second hospital day nor six days later neutralized EMC virus. This result indicates that the rise in antibody in the patients serum was not a non-specific toxic effect. In order to determine if there was an active viral infection at the time of autopsy, frozen sections of the brain, heart and pancreas were stained with FITC-labeled anti-Coxsackievirus B4 antibody. Examination of brain sections revealed viral antigens in a small number of isolated neurons and occasionally in endothelial cells lining small arteries. Out of 40 sections examined, only 7 showed immunofluorescent-positive cells. As a control, sections immediately before and after each section showing immunofluorescent-positive cells were stained with FITC-labeled antiserum to Coxsackievirus B3. These sections were all negative. Sections of the heart and acinar pancreas also were negative when stained with FITC-labeled anti-Coxsackievirus B4 antibody. Islet cells sometimes showed slight fluorescence, but the staining was not strong enough to be scored as positive.

When a certain inbred strain of mice (SJL) was inoculated with the human isolate, over 50% of the animals became hyperglycemic with blood glucose levels sometimes exceeding 400 mg/dl. The concentration of insulin in pooled serum seven days after infection was 1.0 ng/ml as compared to 3.6 ng/ml in serum from uninfected animals. In contrast, some inbred strains of mice such as C57BL6, CBA and Balb/c mice were not hyperglycemic under the same conditions. These results indicate that there were genetically determined host differences in the induction of diabetes when mice were inoculated with the human isolate.

To see whether the hyperglycemia was secondary to beta cell damage, mice (SJL) were sacrificed at different times after infection and their pancreas was examined by light microscopy. Inflammation was visible in the islets of Langerhans similar to that seen in the patient's pancreas. The cellular columns of the islets were diffusely infiltrated with mononuclear cells, the normal architecture of the islet was disrupted and focal cellular degeneration was observed. Lymphocytic infiltrates also were prominent in a perivascular distribution immediately surrounding some islets and in scattered foci in the acinar tissue. Sections of the brain were characterized by an acute meningo-encephalitis with foci of neural degeneration in the cerebral cortex and brainstem. To see whether histopathological changes in the islets of Langerhans were due to viral infection, sections of the pancreas from infected mice were stained with FITC-labeled anti-Coxsackievirus B4 antibody. At four days after infection, viral antigens were clearly seen in the cytoplasm of beta cells. Viral antigens also were found in some acinar cells of the pancreas and in some arteries and neurons of the brainstem. Beginning at about seven days after infection, viral antigens were difficult to detect.

Finally, cultures enriched in human beta cells were inoculated with the human isolate and the susceptibility of the beta cells was determined by the double-label immunofluorescent antibody technique. By this technique, human beta cells prepared from the pancreas of a 17-year-old male (HLA type A2, A33, B15, B21) were quite susceptible, and 60 hours after infection, approximately 37% of the insulin-containing beta cells showed viral antigens.

At present, we do not know the frequency of diabetogenic variants of Coxsackieviruses in the human population. However, it is evident that diabetes is not a common sequela of Coxsackievirus B4 infection, since antibody to Coxsackievirus B4 is present in about half the population, whereas juvenile diabetes develops in less than 0.1 percent of the population. Moreover, it is not known whether the present case is the very rare exception in which the patient became diabetic because of the presence of a receptor for the virus on his beta cells or if he had a specific immune deficiency to the virus or because of other factors. Finally, juvenile diabetes may not be a single disease, but several diseases with different causes. If viruses produce diabetes, they may be only one of the causes, and more than one virus or virus group may be involved.

Reovirus type 3. An in vitro model for culturing pancreatic beta cells was used to test the capacity of reovirus type 3 to infect human beta cells. Antibody to reovirus type 3 was labeled with fluorescein and antibody to insulin was labeled with rhodamine. By use of the double-label antibody technique, it was shown that only 5% of the insulin containing beta cells in culture became infected. However, by repeated passage of the virus in human pancreatic beta cell cultures, the percentage of infected cells increased to 26% and the virus titer increased from 8.4 x 10^4 pfu/ml to 4.9 x 10^6 pfu/ml. Virus assays of the infected cultures showed that virus titers were highest at 72 hours after infection.

Radioimmunoassay for insulin showed that the intracellular immunoreactive insulin decreased beginning at 24 hours after infection, and this decrease roughly paralleled the increase in viral titer, and then leveled off at approximately 50% of that observed in control cultures. In contrast, there was relatively little change in immunoreactive insulin in cultures infected with unpassaged virus. Extracellular immunoreactive insulin levels decreased from 3 days after infection, leveling off at 35% of that seen in the control cultures. These studies show that under in vitro conditions, the infectivity of reovirus for human beta cells is increased by repeated passage and that the infection results in destruction of beta cells and release of insulin.

Fatal viremias: histopathology of the islets. We have examined pancreata from over 250 pediatric autopsy cases with documented evidence of fatal viremia caused by at least 14 different genera of virus from 6 virus families. Islet cell cytopathology and/or insulitis have been found in 20/45 cases with CMV infection, 5/8 cases with Coxsackievirus B infections, 2/14 cases with varicella-zoster infections and 2/45 cases with congenital rubella. Only one case with fatal mumps virus infection was examined and the pancreas appeared normal. These preliminary findings suggest that special attention be directed towards identifying active or reactivated latent viral infections in patients with acute-onset JOD.

IMMUNOLOGICAL STUDIES

Several lines of evidence suggest an involvement of the immune system in the etiology of JOD. One of these has been the demonstration of humoral immunity against islet cells (islet cell antibody, ICA). While the role of these autoantibodies in the pathogenesis of diabetes is not clear, ICA has generated significant interest as a possible marker and biological probe for the study of this disease.

Fresh cryostat sections of blood group O human pancreas have been used almost exclusively as the antigen source for detection of ICA by indirect immunofluorescent techniques. Because of difficulties in obtaining suitable specimens of fresh human pancreas, rigid storage requirements for the frozen tissue, and the relatively poor quality of frozen sections, we looked into the possibility of using permanently fixed, paraffin-embedded tissue.

During this study, we found that fixation with Bouin's solution not only allowed the demonstration of ICA, but that antibodies to insulin (insulin antibody, IA), commonly found in insulin treated diabetics could be detected as well. ICA could be detected with equal sensitivity on fresh, acetone-fixed and Bouin's-fixed pancreas, but not on formalinfixed pancreas. Furthermore, homogenized, fresh, acetone-fixed or Bouin'sfixed pancreas could absorb out ICA while formalin-fixed pancreas could not. Thus, the antigenic specificity of ICA on fresh, acetone-fixed and Bouin's-fixed pancreas was identical. Human IA, on the other hand, could be detected with equal sensitivity on Bouin's-fixed and formalin-fixed pancreas, but it could not be detected on fresh or acetonefixed pancreas. The specificity of this reaction was confirmed by complete absorption of the antibody with insulin. ICA was not affected by insulin absorption.

Titration of guinea pig anti-procine insulin IgG on each of the pancreas preparations suggests that the lack of sensitivity for IA seen with fresh or acetone-fixed pancreas may be due to a decrease in the amount of insulin in the section. Staining of the insulin-containing beta cells with this antiserum could be demonstrated with up to a 1:4,000 dilution on Bouin's- or formalin-fixed pancreas while dilutions up to only 1:8 could stain fresh or acetone-fixed cryostat sections. The best explanation is that formalin and Bouin's solution fixes insulin in situ. Therefore, virtually all insulin contained in the section would be available for reaction with IA. However, since cryostat sections are made of unfixed pancreas, leaching out of insulin is likely during sectioning, acetone-fixation or incubation of fresh pancreas with antisera.

Since Bouin's-fixed pancreas can detect low titers of IA, it is necessary to absorb all samples with insulin before determinations for ICA. Using this technique we have tested 202 sera for ICA in comparison with fresh pancreas. Serum samples gave identical results on each tissue preparation. The incidences of ICA determined in this way were: JOD at time of onset, 84% (21/25); JOD of less than one year duration, 61.1% (11/18); JOD of greater than one year duration, 30.5% (25/82); maturity-onset diabetes, 0% (0/26); and controls, 0% (0/51).

An important approach to understanding the inter-relationships of genetics, immunologic and other factors in the etiology of JOD is the careful study of families with one or more siblings with this disease. We have investigated 35 families, 31 having one child with JOD and four having two such children.

Incidence of ICA in the juvenile-onset diabetics was 57.9% (22/38). Of 77 initially non-diabetic siblings in these families (ages 2-22), 18 are HLA identical to their diabetic siblings. Five of these 18 (27.8%) were positive for ICA and one of these has developed JOD since the start of the study. The incidence of ICA in siblings sharing one HLA haplotype (haploidentical) was 37.5% (18/48) and that for HLA nonidentical siblings was 18.2% (2/11). Oral glucose tolerance tests in these groups were normal as were HbAIc levels. It is also interesting to note that the incidence of ICA in the parents was 20.0% (14/70).

Although this study is in its preliminary stages, it is becoming clear that ICA is more prevalent in families having one or more diabetic children than in the general population and therefore suggests that ICA may be more related to genetic factors (e.g., HLA types) or possibly to environmental factors (e.g., transmissible agents such as viruses) than to the development of JOD. Follow-up studies on the families are in progress.

Implications for Biomedical Research

There are over one million insulin-dependent diabetics in the United States. This form of diabetes is the most difficult to control and the short and long term side effects are enormous with respect to the patient's health and economics. Circumstantial evidence suggests that viruses play some role in the initiation of this form of diabetes. Identification of diabetogenic viruses and preparation of a vaccine might offer a form of preventive medicine that is not otherwise available for genetically predisposed individuals.

Future Plans

Our future plans for research are aimed at: 1) identifying viruses in the pancreas of humans with acute JOD or fatal viremias without JOD and determining if they are diabetogenic in animal model systems ; 2) identifying the properties of different clones of diabetogenic viruses at a cellular level (in vitro) and at the level of the intact organism (in vivo) which can ultimately be used to study the molecular basis of virus-induced diabetes; 3) identifying inbred strains of animals that are susceptible and resistant to diabetes induced by these viruses with the goal of evaluating the genetic and non-genetic factors controlling susceptibility; and 4) identifying antiviral antibodies and autoantibodies in animals and humans with diabetes mellitus to determine the role of the immune response in JOD.

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4. Onodera, T., Yoon, J.W., Brown, K.S., and Notkins, A.L.: Evidence for a single locus controlling susceptibility to virus-induced diabetes mellitus. Nature 274:693-696, 1978.

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9. Notkins, A.L.: What causes juvenile diabetes. Scientific Am. (in press) 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF** **INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 DE 00094-06 LOM

PERIOD COVERED
October 1, 1978 - September 30, 1979

N01 DE 12405-03
CT 0600114

TITLE OF PROJECT (80 characters or less)
Ulcerative Lesions and Tumors: Aphthous Ulcers - Clinical Trials

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☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☒ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The object of this project is to study the etiology and pathogenesis of mucosal diseases of the oral cavity, with the ultimate goal of treatment and prevention At the present time, emphasis is on recurrent aphthous ulcers. A protocol was completed, and the results strongly suggest that mechanical trauma may be one of the precipitating factors in recurrent aphthous ulcers.

PHS-6040
(Rev. 10-76)

ULCERATIVE LESIONS AND TUMORS: APHTHOUS ULCERS

Background and Objectives

The objective of this project is to study the etiology and pathogenesis of mucosal diseases of the oral cavity, with the ultimate goal of treatment and prevention. At the present time, emphasis is on recurrent aphthous ulcers. Aphthous ulcers are characterized by recurrent, painful, single or multiple ulcers on the moist, movable oral mucosa. The etiology of this disease is unknown, however, there is a strong familial tendency and the oral aphthous ulcers often appear following injury to the mucosa. In addition, physical and emotional stress, decreased serum levels of folic acid, iron, and vitamin B12, as well as auto-immune reactions, have been implicated. Microbiological studies have failed thus far to identify any virus or unusual bacterial flora associated with the lesions. Except for the specific replacement therapy in folic acid, iron, and vitamin B12 deficiency, therapy to date has been directed toward palliation of the discomfort caused by the oral ulcers.

Major Findings and Implications

Relationship of Injury and Tissue Repair to Aphthous Ulcers.

Several preliminary reports appeared in the literature in 1949 implicating localized mechanical trauma in the etiology of oral aphthous ulcers. Investigators in a study comparing the effects of mechanical trauma on the mucosa of aphthous patients with individuals without oral mucosal disease produced oral ulcers in aphthous patients but not the controls. It has been postulated that recurrent oral aphthous ulcerations are an end result common to many etiological factors, provided they are capable of initiating sufficient tissue reaction to produce ulceration and that they are applied periodically to the oral mucous membrane. It also seems probable that aphthous ulcers may occur as the result of one or more precipitating factors superimposed upon a mucosa of limited recuperative or defensive potential - the latter aspect attributed to as yet unknown local or systemic factors.

A protocol entitled "The Relationship of Mechanical Injury to Oral Aphthous Ulcers" was initiated in 1977. The purpose of this study is to ascertain if mechanical injury to the oral mucosa is capable of inducing aphthous ulcers in patients known to have recurrent aphthous ulcers. The buccal mucosal injury is produced by inserting and leaving a surgical suture in the mucosa for 3 days or placing a small portion of the mucosa in a surgical clamp for one second. These procedures are done after local anaesthesia has been administered in the operative area. The results are compared with the findings in a matched control group of individuals free of oral ulcerative disease. The study is ongoing at the present time with a total of 30 patients; 20 aphthous and 10 controls having been completed.

The results of the study in 30 patients are as follows: 1) Oral ulcers developed in 8 (40%) of the 20 aphthous patients at one or more sites of induced mucosal injury; 2) the average duration of the experimentally-produced ulcers was 5 days which compares favorably with the usual 7 - 14 day duration span of spontaneous oral aphthous ulcers; 3) the average total size and the average maximum pain also were within the usual limits of spontaneous oral aphthous ulcers; and 4) no ulcers formed in the 10 control subjects, however, a 1 + tissue reaction (swelling and redness) was noted in 3 control subjects.

These findings are significant in the investigations into the etiology of aphthous ulcers because a majority of the naturally occurring oral aphthous ulcers appear at the site of a mucosal injury. Injury to the oral mucosal may allow entry of an agent or agents that initiate ulcer formation. Alternatively, the oral mucosa of aphthous patients responds in an unusual manner to injury, resulting in an expansion of the injury site. A combined immediate and delayed hypersensitiviy reaction to the penetration or release of a foreign antigen is a distinct possibility.

Transmission Experiments

During the past several years, we have been investigating the possible viral etiology of recurrent aphthous ulcers, Behcet's syndrome, Sjogren's syndrome and other dermatologic diseases by attempting to detect the presence of viruses in specimens collected from patients with these diseases. The emphasis of these studies in the past has consisted of efforts to demonstrate viruses *in vitro*, in small laboratory animals and in embryonating hen's eggs. During the past two years, we have extended these investigations. Biopsy specimens obtained from patients have been inoculated into seven primates in an attempt to transmit a slow, latent or persistent virus. These primates are being monitored for the development of clinical disease and for abnormalities in clinical and immunological laboratory assays. The animals have not developed clinical disease as of yet and, therefore, are maintained in order to be observed for late disease development.

Future Plans

During the next year, new investigations into the pathogenesis of recurrent aphthous ulcerations will be initiated with particular emphasis on immune defects and the effect of various treatment modalities on the precipitating factors in recurrent aphthous ulcers.

Publications

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2. Graykowski, E.A., et. al.: Appendix II, Aphthous Stomatitis-Behcet's Syndrome Workshop. Treatment of recurrent aphthous ulcerations. _J. Oral Path._ 7:439-440, 1978.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 DE 00123-06 LOM

PERIOD COVERED
October 1, 1978 - September 30, 1979

TITLE OF PROJECT (80 characters or less)

Herpes Simplex Virus: Latency

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The object of this project is to study the pathogenesis of latent herpes simplex virus infection in both peripheral ganglia and in brain. Biological studies are designed to elucidate factors that induce virus reactivation. Molecular studies are concerned with the state of the latent viral genome, the extent of viral expression, and the mechanism by which latency is established and maintained.

PHS-6040
(Rev. 10-76)

HERPES SIMPLEX VIRUS: LATENCY

Background

Recurrent disease is the main clinical problem in herpes simplex virus (HSV) infection. After the primary infection which usually occurs in childhood, the virus remains latent for years in sensory and autonomic ganglia. At irregular intervals, HSV is reactivated in ganglia. Neural shedding of the reactivated virus ultimately leads to HSV replication in the skin and a clinically detectable recurrent lesion.

Recurrences occur in many sites including the lip and oral cavity, the corneal epithelium, the skin, and the genitalia. About one-third of the adult population suffers from recurrent lip lesions (herpes labialis). Although less common, corneal recurrences (herpes keratitis) are the most common non-traumatic cause of blindness in young adults in the United States. Both lip and eye herpetic infections are usually caused by type 1 HSV. This virus is also the etiological agent of one of the most common of the sporadic encephalitides. Permanent neurological sequelae or death usually result from HSV encephalitis. A related virus species, type 2 HSV, causes aseptic meningitis and most cases of primary and recurrent genital herpetic infections. These genital infections cause a great deal of suffering in terms of discomfort, very frequent recurrences, and venereal spread. In addition, newborns who contract the virus in the birth canal develop a generalized infection which is usually fatal. Finally, there are seroepidemiological data that suggest that genital HSV-2 infection may play an etiological role in cervical carcinoma.

Three general strategies of anti-HSV therapy can be employed: prevention of ganglionic infection, treatment of the productive epithelial infection, and treatment at the latent stage to prevent recurrences. Previous annual reports have discussed prevention by prior immunization and topical drug application. Both of these treatments in experimental animals can block the ganglionic infection under certain circumstances. Similarly, some success has been achieved in man with drug treatment of productive HSV infection, especially encephalitis and keratitis. In contrast, very little experimental work has been done to develop the third strategy: the prevention of reactivation. This strategy, discussed in last years annual report, was investigated by testing antibody, interferon, and drugs in an in vitro reactivation model. Although reactivation was prevented by drugs that blocked the HSV DNA polymerase, the continual presence of drug in the culture media was needed (i.e., the latent infection was not eradicated).

The development of a rational therapy to prevent reactivation must wait for a fuller understanding of latency. To this end, our continuing work in the last year has been mainly concerned with the mechanism of latency and reactivation. Findings in four aspects of this work will be discussed: (1) reactivation of virus by immunosuppressive agents and epithelial irritants, (2) the effect of anti-HSV antibody on the productive infection and the maintenance of latency, (3) the characterization of the latent state in molecular terms, and (4) the demonstration of HSV DNA in brain stem and cerebral hemispheres at the latent stage.

Major Findings

Reactivation by Immunosuppressive Agents and Epithelial Irritation. In the experimental system, HSV is inoculated on the skin surface. The virus enters nerve terminals and is transported in axoplasm to the nerve cell body in sensory ganglia. Then an acute or productive ganglionic infection takes place. After about 2 weeks, the infection passes from the acute to the latent stage. During latency, infectious virus can no longer be recovered in cell-free ganglionic homogenates and viral antigens cannot be detected by immunofluorescent techniques. Yet, it is clear that virus or the viral genome is present in ganglia because HSV can once again be demonstrated after explantation of ganglia in tissue culture. These conditions of a negative homogenate and a positive explant culture essentially define the latent state.

Reactivation refers to the process in which the constraint on HSV replication is removed and virus synthesis resumes in ganglia. Reactivation can be detected directly by recovery of infectious virus in the ganglia or indirectly by recovery of HSV at the epithelial surface. In our experimental model, reactivation as detected by the homogenate method does not occur spontaneously. However, as reported in last year's annual report, reactivation could be induced by treatment of latently infected mice with the immunosuppressive agents cyclophosphamide and x-irradiation. This year, we were also able to induce reactivation with systemic prednisone, but in only 7% of the mice. In these studies, mice were treated with 400 mg/kg i.p. twice daily for 7-10 days. Treatment was initiated about 2 months after corneal inoculation, and reactivation was detected by recovery of infectious virus from cell-free ganglionic homogenates after 7-10 days of treatment. Prednisone in this dose abolished the response of spleen cells in cultures to stimulation by phytohemagglutinin and reduced the spleen weight to about 20% of control. All three of the immunosuppressive agents produced a strong lympholytic effect in mice. However, systemic treatment of mice with lysates from x-irradiated spleen cell cultures failed to provoke reactivation. Negative results were also obtained with systemic LPS (bacterial lipopolysaccharide), and antilymphocyte serum administered i.p. induced reactivation in only 1 of 100 mice.

During the last year, we have also induced reactivation by epithelial irritation. In these studies mice were inoculated by the lip route. Two months after inoculation, dry ice was applied daily to the lips for about 10 seconds. After 7-10 days of this treatment, reactivation could be detected in about 8% of 77 mice from three separate experiments.

Reactivation was detected by recovery of virus from cell-free homogenates of lip or ganglion tissue. Virus or the viral genome could not be detected in lip tissue of untreated mice either by explantation or by a hybridization technique for HSV DNA. It seems likely that the lip irritation sets up a neural signal that in some way triggers virus replication in ganglia. The nature of the signal and the mechanism of reactivation have not yet been established.

Effect of Antibody on the Productive and Latent Stage. These reactivation studies were time consuming since treatment must be delayed until the latent stage. To provide a rapid method to screen a variety of agents, passively immunized mice were used. In these studies, mice were given high-titered rabbit anti-HSV serum 3, 48, and 96 hours after bilateral corneal inoculation with HSV. At the same time, experimental mice were treated (e.g., with cyclophosphamide). The control group was inoculated and passively immunized, but given no treatment. Two and 4 days after inoculation, ganglia explants and homogenates were assayed in the experimental and the control group.

The effect of passive immunization is to bypass the acute stage of the ganglionic infection without actually preventing the infection. These results (i.e. negative homogenate and a positive explant culture) are the same conditions that functionally define the latent stage (see previous section). The acute stage is bypassed in most (about 80%) but not all of the control mice at both 2 and 4 days after inoculation. With cyclophosphamide treatment, however, the acute stage was bypassed in only 21% of mice two days after inoculation and 13% of mice four days after inoculation. This result, therefore, indicates that cyclophosphamide increases the degree of virus replication in ganglia. In contrast, no change from control was seen with LPS, and only a slight change was seen with lip irritation (in lip inoculated mice). Additional agents will be tested in this system to determine if all agents that induce reactivation also enhance virus recovery in the first week after inoculation of passively immunized mice.

To study the effect of anti-HSV antibody on latency, serum neutralizing antibody was measured various times after corneal or lip inoculation. Two groups of mice were used: passively immunized mice given rabbit anti-HSV serum i.p. 3,48,96 and 144 hours after inoculation and control mice given non-immune serum on the same schedule. In the control mice the serum antibody titers were between 8 and 16 NU 7 days after inoculation; 16-32 NU one month after inoculation; and 32-128 NU at 2,3, and 4 months after inoculation. In contrast, antibody titers in the passively immunized mice were 128-256 NU 7 days after inoculation; 8-16 NU one month after inoculation; and < 8 NU 2,3, and 4 months after inoculation. Despite the serum antibody titer of < 8 NU, the infection was still in the latent stage in the passively immunized mice 4 months after inoculation (i.e. negative homogenate, positive ganglion explant cultures). This result indicates that serum neutralizing antibody does not play a critical role in maintaining the infection in the latent stage. The precise biological mechanism that maintains latency has not as yet been defined.

Molecular Characterization of the Latent Stage

A. State of the viral genome during latency. The major thrust of our work is directed to the analysis of the physical state of the HSV viral genome during latency. The experimental approach chosen is directed to the detection of viral DNA sequences covalently joined to cellular DNA as a test for integration. To this end, DNA from latently infected ganglion is digested with restriction enzymes, and the fragments generated are separated by agarose gel electrophoresis on the basis of their molecular weight. The DNA is then blotted onto nitrocellulose paper and hybridized to ^{32}P-labeled HSV DNA. All of the viral restriction fragments will appear at their true molecular weight only if the viral DNA is not covalently joined to cellular DNA sequences. The molecular weights of the viral DNA fragments are known and can always be determined by inclusion of the proper markers in the same gels. If one or more viral DNA fragments display an increase in molecular weight, it will be indicative of covalent joining of the virus DNA to the cell DNA, and thus of integrated viral sequences.

This experimental design, although simple, has been plagued with unpredictable problems. First, since it was impractical to look for the whole of the HSV-genome, certain sequences of interest had to be selected. The sequences at the termini of the viral DNA were chosen, since the simplest hypothesis would be that of terminal integration. A terminal fragment (K) generated by the restriction enzyme Eco RI was chosen because of its ease of purification and higher relative abundance in the genome. It soon was found that the viral DNA termini are heterogeneous to the extent that after passage, three K fragments were distinguishable, differing by no more than 200 base pairs from each other. This fact would have posed an inadmissible source of variability in any experimental results; thus K itself had to be fragmented by a second enzyme (Bam H1). Two fragments of K were purified: a terminal K-Bam (B_1) of 3.2-3.4 kilobase pairs (kbp) and an internal K-Bam (B_2) of 1.5-1.7 kbp. B_1 but not B_2 is heterogeneous, showing that the heterogeneity occurs at the end of the molecule. Both fragments are being used as probes for the detection of their complementary sequences in the ganglion DNA.

Secondly, it was found that the viral DNA sequences present in trigeminal ganglia were undetectable in blot hybridization if the ganglion DNA was simply restricted and run in agarose gels. The reason was 2-fold: first, the low representation of viral DNA in the ganglion DNA (0.01 genome equivalents per cell, equivalent to 1 ng of viral DNA per 350 ug of cell DNA); and second, the limited amount of DNA that could be loaded onto a gel. It was necessary to enrich the ganglion DNA for viral sequences. Several techniques, including CsCl and KI buoyant density gradient fractionation, thermal elution from hydroxyapatite, and preparative gel electrophoresis were unsuccessful in enriching reconstitution mixtures of DNA without at the same time leading to shearing of DNA into small fragments. This was an undesirable result. It was finally possible to enrich for viral sequences by a combination of affinity chromatography in phenyl-red bisacrylamide (G-C specific) and reverse-phase chromatography in RPC-5. RPC-5 fractionates DNA on the basis of fragment size, G-C content, base stacking and an array of other as yet unknown factors. It is common to obtain a 4-fold enrichment in phenyl-red columns and 10-fold in RPC-5 although the values vary from batch to batch of resin. However, the high pressure and high ionic strength needed to run RPC-5 has led to two other problems, viz. fragmentation of the high molecular weight DNA species and re-hybridization of fragments by tails made by the restriction enzymes. High-pressure columns had to be redesigned eliminating all fittings with pore size smaller than 60 microns and substituting them with hand-made porous polyurethane fittings. Techniques had to be developed to denature spurious tail-to-tail hybridization without denaturing the whole of the DNA fragment.

Thirdly, non-specific hybridization to mouse DNA in blots and to the nitrocellulose support had to be reduced to practically zero levels in order to be able to detect picogram quantities of viral DNA. After several unsuccessful trials, it was found that inclusion in the hybridization mixtures of carrier DNA's from mouse, rabbit, dog, cow, sheep, and salmon sperm would completely eliminate non-specific hybridization. A graded series of washings of the blots at high temperature with decreasing concentrations of SSC-buffer in the presence of sodium pyrophosphate would eliminate sticking of the probe to the nitrocellulose membrane.

At this point, it is possible to detect 100 picograms of HSV DNA fragmented with Eco RI (smallest fragment 1 picogram) in a background of 50 ug of carrier DNA. It should be possible to have a definite answer to the integration question very soon, now that most of the unpredicted technical problems have been eliminated.

B. Viral expression during latency. In order to characterize in molecular terms the latent stage of HSV infection of mice, it is important to determine whether expression of the viral genome occurs, and if so, to what extent.

Published work from this Laboratory reported detection of the viruscoded thymidine kinase (tk) enzyme in extracts prepared from dorsal root ganglia taken from latently infected mice up to a maximum of 60 days post HSV inoculation. In order to extend these studies, a highly sensitive assay, utilizing very high specific radioactivity, 5-[^{125}] iododeoxycytidine (5-^{125}IdC) as enzyme substrate was introduced last year.

As discussed in last year's annual report, in the presence of tetrahydrouridine phosphorylation of IdC is blocked in extracts prepared from uninfected cells, but can occur via the viral Tk enzyme in extracts prepared from HSV infected cells. Immune IgG, but not non-immune IgG, blocks this reaction in infected cell extracts.

During the past year, extensive studies of Tk activity in mouse trigeminal ganglia latently infected with HSV following corneal inoculation, have been undertaken. Ganglia were assayed either individually or as pools taken at different times after inoculation up to a maximum of 208 days post inoculation.

In the course of these studies, a number of unforeseen problems were encountered. First, the radio-labeled substrate (5- ^{125}IdC) was found to be unstable and decomposed to produce an unidentified breakdown product, which though it did not function as a substrate for any enzyme in the ganglion extracts, did produce high backgrounds in the assay. A purification system for the substrate was successfully developed and used to purify the substrate about every two weeks. Also a thin layer chromatography system, capable of separating the reaction product (IdCMP) from the impurity (X) and unreacted substrate (IdC) was used to determine the degree of phosphorylation of the substrate without interference from any breakdown products.

The second and major problem was that addition of highly purified IgG (immunoelectrophoretically pure) prepared from immune rabbit serum raised against HSV infected primary rabbit kidney cells resulted in stimulation of the Tk activity in ganglion extracts prepared from latently infected mice and in some cases from uninfected mice, but never from cells productively infected with HSV. Since the source of the Tk enzymes is a crude cell extract, the assay system is necessarily complex and numerous control experiments were undertaken to establish the reason for the stimulation. These experiments suggested that the stimulation was not due to "factors" in the IgG preparations used, but was probably due to some immunologic cross-reactivity with one or more of the cellular mitochondrial Tk isoenzymes. This problem was largely overcome by using heat-inactivated Staphylococcus aureus bearing protein A as an immunoadsorbent for immune complexes formed when ganglion extracts were reacted with normal or immune IgG prior to being assayed for Tk activity. Also, additional sera raised in rabbits against the highly purified HSV Tk enzyme were obtained and used in these studies.

The results of these studies showed that the HSV specific Tk enzyme could only be detected during the acute phase of the infection of mouse trigeminal or dorsal root ganglia but not during the latent phase of the infection. Exactly the same result was obtained using a modified version of the standard assay system (using [^{3}H]-Thymidine as substrate) capable of detecting the HSV Tk activity arising from only one in a thousand productively infected cells. The results of this study are in agreement with data reported in last year's annual report, showing that there is a block in transcription of the HSV genome at the latent stage of the infection.

Currently, other techniques are being developed in the Laboratory to search for HSV antigens other than the viral Tk in latently infected ganglia. These techniques involve isolating HSV antigens on immunoadsorbents and analyzing them on denaturing polyacrylamide gels. Preliminary experiments on latently infected ganglia which have been reactivated *in vitro* for up to 48 hours show that HSV antigens are present in very small amounts. This suggests that if expression of some HSV antigens (e.g., immediate early polypeptides) occurs during the latent phase of the infection, their detection would require extremely sensitive techniques.

HSV DNA in the Central Nervous System. Up to this point our work has concentrated on HSV infection in ganglia of the peripheral nervous system. In the last year, we initiated studies of latency in the central nervous system (CNS).

At 6 days after corneal inoculation, HSV was demonstrated in homogenates prepared from 18 of 20 brain stems and 6 of 20 cerebral hemispheres. In the same group at the latent stage (5-8 weeks after inoculation), homogenates were negative in 20 mice. But unlike ganglia, explants at the latent stage usually failed to yield virus (positive explants in 0 of 10 brain stems and 1 of 10 hemispheres). Since the mortality in these mice was $< 5\%$, it is clear that the animals survived a productive CNS infection. The negative explants may mean that HSV is eliminated at the productive stage or that HSV cannot be readily reactivated from the CNS by explantation techniques.

To settle this question, hybridization techniques were used to assay for HSV DNA sequences, both at the acute and latent stage. Since viral sequences, if at all present, would be expected in a lower relative abundance than in ganglia, it was important to enrich the brain DNA for viral DNA. The technique employed was affinity chromatography on phenyl-red substituted bisacrylamide. The phenyl-red ligand binds G-C pairs in DNA, that can then be eluted with a gradient of $NaClO_4$; a tighter binding requires higher periodate concentration and is indicative of higher G-C content. Since HSV has 25% higher G-C than mouse DNA, it could be separated from cell DNA sequences using this technique. The complementary dye-substituted support, malachite-green bisacrylamide, is specific for A-T rich DNA and was also used to enrich for viral DNA. Both techniques gave on reconstitution experiments enrichment factors of 5-10 fold, which were considered sufficient for the experiments to be attempted.

Thirty animals were sacrificed at both acute and latent phases of the infection, and brain stems and cerebral hemispheres from these mice were pooled. DNA was extracted and enriched for viral DNA sequences by the techniques just described. Hybridizations were done using high specific activity ^{32}P-labeled viral DNA. Detection of the hybrids was by hydroxyapatite chromatography. The pool of thirty acute brain hemispheres was shown to have approximately 0.29 viral DNA equivalents per diploid cell; in the corresponding pool of thirty acute brain stems 0.13 viral

DNA equivalents per diploid cell were detected. In pools of thirty hemispheres removed at the latent state, 0.017 HSV genomes per cell were found; the corresponding brain stems removed during the latent stage had a very similar number of 0.016 viral genomes per cell.

In order to determine whether only one or a few of the brains comprising the pool had viral DNA, the experiments were repeated with individual brains (hemispheres or stems) being singly analyzed. Tissue DNA was extracted separately from latently infected animals and was hybridized to ^{32}P-labeled HSV DNA. In these tissues, viral sequences were found in one out of ten hemispheres, at a level of 0.05 genome equivalents per cell, and in five out of ten stems, at a level varying from 0.035 to 0.003 equivalents per cell.

We are in the process of repeating this experiment. A group of infected mice is being followed both through the acute and latent stages by infectivity assays and molecular hybridization studies. Ten acute hemispheres and stems have been analyzed by hybridization so far and they all show the presence of viral DNA sequences. Brains from latently infected mice will be analyzed in the forthcoming months.

Future Studies

Immediate studies will be directed to continue two major projects: determination of the physical state of the viral genome during latency, and presence of viral sequences in the brain. It is foreseeable that if the viral genome is shown to be integrated, we would attempt cloning the cellular joint sequences in a suitable vector, by recombinant DNA techniques. This would allow for a study of the make-up of these sequences. A major emphasis will be to determine whether integration occurs at unique points of the cellular chromosome and to discriminate the four possible isomers of the HSV genome amongst the integrated sequences.

If the presence of viral DNA in the CNS is confirmed, we would like to extend our search for HSV sequences to the naturally occurring infection in humans. If it can be shown that viral latency is not confined to the trigeminal ganglion, herpes simplex would become a reasonably good candidate as agent for various neurological diseases of unknown etiology. The fact that, at least in mice, viral sequences can be found in the brain, yet virus cannot be readily reactivated by explantation techniques, may indicate that the whole genome is not present in the affected tissues. It would then be important to determine which sequences are there and to what extent they are or are not expressed.

Publications

1. Asher, L.V., Walz, A., and Notkins, A.L.: Effect of immunization on the development of a latent ganglionic infection in mice challenged intravaginally with HSV-1 and HSV-2. J. Obs. & Gyn. 131:788-791, 1978.

2. Puga, A., Rosenthal, J.D., Openshaw, H., and Notkins, A.L.: Herpes simplex virus DNA and mRNA sequences in acutely and chronically infected trigeminal ganglia of mice. Virol. 89:102-111, 1978.

3. Openshaw, H., Asher, L.V.S., Wohlenberg, C.R., Sekizawa, T., and Notkins, A.L.: Acute and latent infection of sensory ganglia with herpes simplex virus: Immune control and viral reactivation. J. Gen. Virol. 44:205-215, 1979.

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6. Openshaw, H., Puga, A., and Notkins, A.L.: Latency and reactivation of herpes simplex virus in sensory ganglia of mice. In Virus Lymphocyte Interactions: Implications for Disease. Edited by Max Proffitt, Elsevier North-Holland, New York (in press) 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF** **INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 DE 00200-03 LOM

PERIOD COVERED October 1, 1978 - September 30, 1979

TITLE OF PROJECT (80 characters or less)

Ulcerative Lesions and Tumors: Papillomas of Oral Cavity

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☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Human cutaneous and oral cavity papillomas are being examined for the presence of human papilloma virus (HPV) by an antiserum which is cross-reactive with an antigen present in all papillomaviruses. Fluorescent antibody (FA) tests are being done on frozen sections of wart tissues and peroxidase - antiperoxidase (PAP) tests are being done on sections of formalin-fixed tissue.

PHS-6040
(Rev. 10-76)

ULCERATIVE LESIONS AND TUMORS: PAPILLOMAS OF THE ORAL CAVITY

Background

The papillomaviruses cause cutaneous and mucosal papillomas in man and animals. The human papillomavirus (HPV) is the only known virus that unequivocally causes neoplasia in man. Although most of the neoplasms known to be induced by HPV appear to be benign and many spontaneously disappear, occasional oral and cutaneous papillomas will undergo malignant transformation into squamous cell carcinomas. During the last few years at least five different HPV genotypes (HPV-1, HPV-2.... HPV-5) have been identified in cutaneous papillomas. These genotypes are not immunologically cross-reactive and can be distinguished from one another by immunologic tests as well as by DNA homology studies. Recent studies, using tests to distinguish between the HPV genotypes, suggest that each type of HPV appears to be the causative agent of specific clinical types of papillomas. A greater variety of clinical types of papillomas occur in the oral cavity (verruca vulgaris, verruca plana, focal epithelial hyperplasia (FEH), condyloma accuminatum, oral papillomas and laryngeal papillomas) than anywhere else in the body. It is highly probable that some of these papillomas are caused by HPV other than HPV types 1-5.

Objectives

The objectives of this research study are to obtain representative samples of cutaneous and oral cavity papillomas, determine which lesions have an HPV etiology, and if they do, identify the types of HPV.

Major Findings

We have collected FEH from native Americans in the southwestern United States, laryngeal papillomas from otolaryngology clinics specializing in this disease, and other oral cavity papillomas from dentists in this area. We also obtained cutaneous papillomas from University wart virus clinics, podiatrists, and dermatologists. Most of the papillomas that we are working with have been easily identified clinically and the diagnosis confirmed pathologically. Each fresh specimen has been divided up for electron microscopy, immunology, and molecular virology studies for identification and characterization of HPV.

All of the cutaneous papillomas in our study were either verruca vulgaris or verruca plantaris. When examined with the electron microscope, approximately one-half of these warts contain HPV particles. We determined the type of HPV in many of these virus-positive lesions by either immunologic studies using specific antisera against HPV-1 or molecular virologic studies on purified HPV. Almost all of the plantar warts contained HPV-1 and most of the common warts contained HPV-2. We then turned our attention to the oral cavity papillomas. Only one of the oral cavity lesions, a verruca from the mucosal surface of the vermillion border, was virus-positive by electron microscopy. It did not contain HPV-1 as determined by immunologic studies with specific antisera prepared against HPV-1. Moreover, molecular hybridization studies on DNA extracted from many of the oral cavity lesions using HPV-1 DNA as the probe, showed that there was no evidence of HPV-1 DNA or related HPV DNA intermixed with cellular DNA.

The major limitation in this study has been the difficulties encountered in trying to identify HPV other than HPV-1 in oral cavity papillomas. We started out using electron microscopy as a general screening technique for identifying HPV-positive lesions. This is a laborious process and because of the possibility of sampling error, small numbers of HPV-positive cells could easily be missed. We also immunized rabbits with individual HPV isolates to produce specific antisera against HPV. However, since plantar warts were the only papillomas that yielded enough HPV for immunization, all of the antisera that we collected was reactive only with the outer shell or capsid of the plantar wart virus, HPV-1. Because this antiserum had limited usefulness as a screening technique for detecting papillomas other than the plantar wart, we decided to try another approach using HPV-1 as an immunogen. We disrupted HPV-1 with a detergent and used this preparation to try and produce antibodies against the internal components of HPV-1 with the thought that maybe one of these components was common to all types of HPV. Antiserum prepared by immunization of rabbits with detergent-disrupted plantar wart HPV was cross-reactive with all virus-positive papillomas from humans and other species (cow, dog, deer, horse) by both fluorescent antibody (FA) tests on frozen sections of papilloma tissues and peroxidase-antiperoxidase (PAP) tests of sections of formalin-fixed tissue. This finding provided us with a method other than electron microscopy to use as a screening technique to detect HPV regardless of type. Since one of these immunologic tests (PAP) can be performed on formalin-fixed paraffin-embedded tissue, it can be used to test for presence of HPV in papillomas stored in paraffin blocks.

Significance

Identification of the type(s) of HPV in mucosal papillomas is necessary to help prepare a rational therapeutic approach, such as vaccination, for lesions like laryngeal papillomatosis which is often a life threatening disease. It also would help us to understand the epidemiology of lesions such as might occur with oral-oral, oral-skin or oral-genital spread of different HPV types. Once we have learned how to identify the types of HPV in oral lesions, we may be able to identify HPV in other benign, and perhaps, malignant, papillary lesions which occur in different epithelial sites throughout the body.

Future Studies

We have just started screening large numbers of formalin-fixed, paraffin-embedded oral cavity papillomas for the presence of HPV. We are using antiserum directed against the common papillomavirus antigen, using the PAP technique. After identifying the lesions that most often contain significant amounts of HPV, we can then concentrate on collecting these papillomas from various clinics for the purpose of virus isolation. HPV can then be isolated from them and the HPV DNA can be cloned in bacterial plasmids. Large quantities of HPV DNA can then be obtained from oral cavity lesions for molecular virologic studies (endonuclease restriction studies and molecular hybridization studies).

Publications

Jenson, A.B., Openshaw, H., Hooks, J.J., Puga, A., and Notkins, A.L.: Herpes and other virus-induced oral diseases. In Current Advances in Oral Biology, edited by Dr. H.C. Slavkin (in press) 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 DE 00219-03 LOM

PERIOD COVERED

October 1, 1978 - September 30, 1979

TITLE OF PROJECT (80 characters or less)

Herpes Simplex Virus: Cell-Mediated Immune Mechanisms and Interferon

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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Laboratory of Oral Medicine

SECTION

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5.30	2.75	2.55

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☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The objective of this research project is to determine the role of viruses and interferon in immunologic and immunopathologic processes.

The observation that immune interferon could be produced by peripheral blood leukocytes in vitro on an immune specific basis suggested that it might be produced in vivo in various autoimmune disorders. We found immune interferon in the sera of patients with active immunologic diseases, such as systemic lupus erythematosus, rheumatoid arthritis, scleroderma and Sjogren's syndrome. Furthermore, the presence of interferon was correlated with clinical disease activity.

It is possible that the production of interferon may contribute to immunologic aberrations in autoimmune diseases and also protect the already compromised host from viral infections.

PHS-6040
(Rev. 10-76)

CELL-MEDIATED IMMUNE MECHANISMS AND INTERFERON

Background and Objectives

This research project is divided into three components: 1) the role of viruses and interferon in immunological processes, 2) chemotactic and mitogenic antibodies, and 3) hybridomas and monoclonal antibodies to viral antigens. Each project will be discussed separately.

INTERFERON

Interferon was first recognized as a soluble antiviral glycoprotein induced by viruses. Recently, it was shown that there are at least two types of interferon. Type I, virus-induced interferon is produced by both non-lymphoid and lymphoid cells in response to viruses. Type II or immune interferon is a mediator of cellular immunity, a lymphokine and is produced by sensitized lymphocytes in response to specific antigens. In addition to their antiviral activity, both types of interferon can affect antibody production and cell-mediated immunity.

The objective of this research project is to determine the role of viruses and interferon in immunologic and immunopathologic processes. In the last two annual reports, we described our experimental data on the role of viruses and interferon in immediate hypersensitivity (allergic) reactions.

Histamine and other mediators of anaphylaxis are released when IgE on the membrane of human peripheral blood basophils interacts with specific allergens (e.g. ragweed antigen). This release of histamine from human basophils by allergens has proved to be a good in vitro model for immediate hypersensitivity (allergic) reactions. Using this model, we found that incubation of human leukocytes with certain viruses before challenge with ragweed antigen enhanced the release of histamine. Further studies showed that this enhancement of histamine release was in fact due to interferon induced by viruses. Moreover, an induction period and new RNA synthesis was required for interferon to exert its effect.

Interferon also can be induced by stimulation of leukocytes from an immune host by specific antigens. In addition to the release of interferon, the stimulation of immune leukocytes by specific antigens results in the release of a variety of other biologically active mediators (lymphokines). Last year, we showed that immune-specific stimulation of leukocytes results in the release of soluble mediators that are capable of enhancing IgE-mediated histamine release. This data suggested to us that interferon also might be produced in patients with diseases involving an immune response. Our studies have focused on the production and role of immune interferon in human immunopathalogic processes. To investigate this problem, we have (1) measured serum levels of interferon in patients with autoimmune diseases, and (2) analyzed the relationship between interferon activity and disease activity.

Major Findings

Interferon in the serums of patients with autoimmune diseases. During the past year we have looked for interferon in the sera of patients with autoimmune disease, such as systemic lupus erythematosus, rheumatoid arthritis, scleroderma and Sjogren's Syndrome. These systemic diseases share certain clinical characteristics and are all mediated by aberrations in immunological functions. Systemic lupus erythematosus is a multisystemic disease associated with autoantibodies and circulating immune complexes. The disease is characterized by widespread inflammatory changes in connective tissues, vessels and serosal surfaces. Renal disease is common. Rheumatoid arthritis is a systemic disease with subacute or chronic inflammatory arthritis that affects mainly peripheral joints. Scleroderma, which occurs less often, produces varying degrees of vascular change, fibrosis and inflammation of skin and internal organs. Sjogren's Syndrome is a chronic, inflammatory disease characterized by dryness of the mouth, eyes, and other mucous membranes.

We detected interferon more frequently in the sera of patients with these autoimmune diseases than in the sera of healthy controls. Interferon was found in the sera of 46 per cent of patients with systemic lupus erythematosus, 50 percent of patients with rheumatoid arthritis, 60 percent of patients with scleroderma and 27 percent of patients with Sjogren's syndrome. The highest levels of interferon (¯ 32 Units/ml) were found in patients with systemic lupus erythematosus.

Relationship between disease activity and interferon in systemic lupus erythematosus. Since patients with systemic lupus erythematosus have flare-ups and remissions, these patients were separated into groups with and without active disease. We found interferon in the sera of 71 percent of patients with active systemic lupus erythematosus but in only 21 percent of those in remission.

These results suggest that there is a relationship between the presence of interferon and clinical disease activity. In order to further elucidate this relationship, we obtained serial serum samples from nine patients with systemic lupus erythematosus and tested these sera for the presence of interferon. The first 5 patients showed clinically active disease at some time during the study. When symptomatic, these patients showed interferon levels ranging from 16 to 128 U per ml. When patients were free of clinically active disease, interferon was detected in the circulation at levels of 8 U per ml or lower. The other 4 patients had clinically inactive disease throughout the observation period (10-20 months). Interferon was rarely detected and never at levels above 8 U per ml.

Antibody to double stranded DNA and low levels of the 3rd component of complement in the sera are both recognized laboratory measures of disease activity of systemic lupus erythematosus. Our studies showed that clinical disease activity was correlated with interferon activity and with antibody to DNA and low serum C_3 levels. The striking correlation with disease activity, in fact, raises the possibility that interferon levels may be a better index of such activity than the laboratory measures now in use.

Characterization of interferon. The antiviral activity in the patients sera was shown to be interferon. The serum samples inhibited replication of vesicular stomatitis virus (VSV) on human cell lines but not on mouse cells. Moreover, the serum samples were not toxic for human cells; an induction period was required for development of the antiviral state, incubation of serum samples with VSV did not result in viral neutralization, and the antiviral activity was destroyed by trypsin. Since immune interferon is inactivated by treatment with acid, whereas standard interferon is resistant to such treatment, the sera were tested with acid. Since acid treatment destroyed the antiviral activity of the patient's sera, we conclude that the interferon was immune interferon.

Significance

Our studies show that immune interferon is produced in patients with immunologically active disease. The role of interferon in these immunological diseases is still conjectural. However, it is known that interferon may alter or modulate immune responses. It is possible that immunological aberrations associated with systemic lupus erythematosus, such as increased antibody levels, autoantibodies, immune complexes, depressed cell-mediated immune responses and kidney disease may in part be mediated by circulating immune interferon.

Clinical disease activity in patients with systemic lupus erythematosus follows a course of exacerbations and remission. Unfortunately, the laboratory measures of disease activity do not always correlate with clinical disease. It is therefore sometimes difficult to carefully evaluate the efficacy of experimental drug treatments in these patients. Our findings suggest that interferon levels may be a better index of disease activity than the laboratory measures now in use.

Interferon is effective as an antiviral agent and defends the host against viral infections. It was recently reported that herpes-zoster virus infections occur less frequently in patients with active systemic lupus erythematosus than in those with inactive disease. This observation might be explained by the presence of interferon in the blood of those with active disease. Thus, production of interferon during the host's immunologic responses to one disease may protect the host from contracting a second (e.g. viral) disease.

Future Plans

The studies reviewed in this report provide insight into the possible mechanisms involved in autoimmune diseases which are characterized by aberrations in immunoregulation. Over the next year, these studies will be continued and expanded. Interferon in the sera of lupus patients will be further characterized and its continued presence in patient sera for prolonged periods of time will be documented. We also plan to evaluate the role of virus infections or interferon production in the exacerbation of disease activity in patients with systemic lupus erythematosus.

Our studies raise the possibility that type II interferon may be present in the peripheral blood of patients with a variety of other immunologic disorders. We are presently investigating this possibility in diseases such as multiple sclerosis, Wegener's granulomatosis, vasculitis and sarcoidosis.

CHEMOTACTIC AND MITOGENIC ANTIBODIES

Background

Mouse epidermal growth factor (mEGF) is a small polypeptide (molecular weight 6,045) purified from the submaxillary gland of adult male mice. This factor, at very low concentration, stimulates epithelial growth in *in vivo* and in organ culture and is mitogenic for many ectoderm and endoderm-derived cells.

Developmental biologists concerned with in vivo control of cell proliferation and differentiation, and pathologists studying wound-healing have been very interested in the role of EGF in the proliferation of different cell types involved in these processes. As discussed last year, the coupling of biologically active molecules (e.g., chemotactic factors) to immunoglobulins, a highly specific carrier, could represent a potent tool for "targeting" these molecules to specific sites in vivo.

Major Findings

Chromatographically purified antibody (IgG) to herpes simplex virus or sheep red blood cells was coupled to mouse epidermal growth factor (mEGF) in the presence of trace amounts of iodinated mEGF, using gluteraldehyde as the coupling agent. Samples were dialyzed and chromatographed on a Sephacryl S-200 column. The ^{125}-mEGF appeared in the same peak as the IgG. This demonstrates that mEGF was coupled to the IgG molecule. The coupled material then was assayed for its ability to stimulate DNA synthesis of human foreskin fibroblasts. Both preparations coupled to mEGF stimulated DNA synthesis. The controls, IgG alone and IgG treated with gluteraldehyde, were devoid of stimulatory activity.

To see if the antibody-binding site of the IgG molecules remained intact after coupling with mEGF, neutralization tests for HSV and hemagglutination tests for SRBC were performed. In both cases, the IgG retained its capacity to bind to antigen.

Significance

The coupling of EGF to specific immunoglobulins may make it possible to target mitogenic molecules to specific in vivo sites. If successful, this may aid in the restoration of damaged tissue as in wound-healing where massive proliferation of epidermal and fibroblast cells is required.

Future Studies

In the future, we hope to determine whether immune complexes composed of EGF coupled to antibody (mitogenic antibody) and bound to antigen are capable of stimulating DNA synthesis and whether other growth stimulating factors can be coupled to immunoglobulins.

HYBRIDOMAS

Background

By conventional immunization methods, it has been difficult to obtain monoclonal antibody to specific antigenic determinants on complex antigens. Recently developed techniques have made it possible to produce antibody of restricted specificity (monoclonal) to certain antigens. For example, P3 x 63 Ag8 myeloma cells [which are resistant to 8-azaguanine due to deficiency of hypoxanthine phosphoribosyl transferase and cannot survive in the media containing hypoxanthine, aminopterine and thymidine (HAT)] is hybridized to primed spleen cells from animals immunized with specific antigens. These hybrid cells can survive in HAT media, they proliferate continuously, and secrete large amounts of homogeneous antibody to specific antigens.

Major Findings and Significance

Over the last eight months, we have established the hybridoma technique in our Laboratory to obtain monoclonal antibodies against different antigenic determinants on viruses such as herpes simplex, Coxsackievirus B4, encephalomyocarditis and reovirus. In our experiments, Balb/C mice were immunized intraperitoneally with increasing doses of virus. On the fifth day, after the last intravenous booster, spleen cells were prepared from the sacrificed mice and fused with P3 x 63 Ag8 meyloma cells, using polyethlene glycol (PEG) as the fusing agent. The hybrids were grown in HAT media for 1-2 weeks and then neutralizing antibody levels were checked from different wells of the hybrid cultures. About 20% of the tested samples produced neutralizing antibody. Wells that produced high titers of anti-viral antibody then were subcultured. After subculturing, neutralizing antibody levels were checked again. About 30% of the subcultures consistently produced antibody. Single clones were isolated by the soft agar cloning method or by the limited dilution method. The production and characterization of monospecific antibodies from these clones are now under investigation. This technique will be of great value in distinguishing various strains of viruses and possibly relating altered antigenicity to virulence.

Publications

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6. Hooks, J.J., D. Benezra, L. Cohen, A. Dattner, B. Detrick-Hooks, T. Lehner, C. Mebus, and H. Openshaw. The classification, pathogenesis and etiology of recurrent oral ulcerative diseases and Behcet's syndrome. J. Oral Path. 7:436-438, 1978.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 DE 00255-01 LOM

PERIOD COVERED October 1, 1978 - September 30, 1979

TITLE OF PROJECT (80 characters or less)

Receptors, Membranes and Diseases

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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TOTAL MANYEARS: 3.60	PROFESSIONAL: 2.10	OTHER: 1.50

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Cell surface receptors for viruses and polypeptide hormones were studied in cells of murine and human origin. The human membrane receptors for Encephalomyocarditis virus were found to be quantitatively and qualitatively different from those on murine cell membranes, although cells of both species are susceptible. Another class of membrane receptors, those for the polypeptide hormone insulin, was studied following infection with Herpes Simplex and other viruses. The number of insulin receptors decreased following herpes simplex and vesicular stomatitis viruses but not after infection with measles and Sinbis viruses.

PHS-6040
(Rev. 10-76)

RECEPTORS, MEMBRANES AND DISEASE

Background

The surfaces of cells are known to possess receptors which determine if and how those cells will respond to viruses, hormones, and other cells. In recent years, the relationship between cellular receptors and disease has become increasingly evident. Most viruses must attach to a cellular receptor in order to infect a target cell, and the absence of an appropriate receptor can render a cell resistant to those viruses. Antibody to hormone and neurotransmitter receptors can produce serious disease as in extreme insulin resistance, Graves disease and myasthenia gravis. Obesity can lead to decreased insulin reception and a mild to severe insulin-resistance.

In recent years, it has become evident that many diseases are associated with specific histocompatibility antigens. The HLA (human) and H-2 (mouse) major histocompatibility loci govern the expression of the strongest and perhaps the most important of the cell surface antigens. Insulin deficiency, diabetes mellitus, multiple sclerosis, and a host of rheumatological disorders have been found associated with increased representation of certain HLA haplotypes. It is not unreasonable to consider that an apparently minor change in one or more cell surface components could lead to altered cellular function and eventually to multi-systemic disease.

We have been investigating the relationship between receptors and disease from several approaches. First, since the susceptibility of a given cell to a virus largely depends upon the presence and characteristics of a cellular receptor for that virus, we have been comparing cells of differing susceptibilities to encephalomyocarditis virus (EMC) with respect to the kinetics of receptor binding. Second, previous work from the Laboratory of Oral Medicine has demonstrated that a single virus can produce dramatically different diseases in different mouse strains. We have been attempting to determine the genetic basis of differential susceptibility by studying the segregation of susceptibility and resistance in crosses of susceptible and resistant strains of mice. Third, some viruses are known to modify the plasma membrane of their host cells. In some cases, viral antigens or new host antigens are displayed, while in others, normally present components are decreased. Therefore, we have been using a selected surface cell receptor, the one for insulin, as a probe of possible cell surface changes in viral infections.

Major Findings

Receptors for EMC. In our initial attempts to study the interaction of EMC virus with its receptors, we made the assumption that the behaviour of EMC would be similar to that of other picornaviruses. In fact, EMC virus proved to be quite different in its binding kinetics and optimum conditions for attachment. In these studies, the cells used were: secondary mouse embryo cells (SME), mouse L-929 fibroblasts, mouse erythroblastoid cells (FLC), HeLa cells, and human and mouse erythrocytes. The virus used was plaque-purified from heart-passaged M-variant EMC, and was designated EMC-108D.

When we initially attempted to measure the binding of EMC to SME cells or L-929 cells at 20°C and 37°C, no attachment was found. Surprisingly, when the binding reaction was attempted at 0°C, the virus rapidly attached to the cells. The binding was specific as judged by the failure of mouse cells to attach poliovirus or inactivated EMC. It was also found that EMC would attach to HeLa cells 5 to 10 times more rapidly than to cells of murine origin. The attachment of [^{3}H]leucinelabeled EMC to HeLa cells was completely blocked by pre-incubation with a 100-fold excess of unlabeled virus.

Others have reported that EMC attaches to and agglutinates human erythrocytes. When both human and murine erythrocytes were tested in our assay, the human cells rapidly adsorbed the ^{3}H-labeled virus; however, no binding of radioactive virus to the murine erythrocytes was observed. When these experiments were performed using plaque assays to monitor the number of infectious units, it was found that both HeLa cells and FLC attached and eclipsed the virus at 0°C. In contrast, human erythrocytes attached virus but did not eclipse it, and murine erythrocytes did neither.

The binding of EMC to murine cells and to HeLa cells proved to be highly temperature-dependent. At temperatures above 10°C, the binding to HeLa cells is appreciably slower, and the binding to L-929 cells is almost completely inhibited. All of the available data indicate that HeLa cells have more receptors per cell for EMC and that these receptors may have a higher affinity for the virus. Mouse cells on the other hand, appear to have fewer receptors per cell, and once bound there is a greater tendency for the virus to elute from the murine cells. Furthermore, the rate of elution from murine cells is increased with increasing temperature to a greater extent than the rate of elution from HeLa cells. We are in the process of determining whether the difference in the number of receptors per cell can completely account for the difference in the kinetics of attachment of EMC to HeLa cells and murine cells, or whether the thermodynamic properties of the receptors are truly different.

Genetics of resistance to virus infection. Earlier work in the LOM showed that C57BL/6J mice were resistant to EMC-induced diabetes mellitus, and that SJL/J and DBA/2J mice were highly susceptible. Furthermore, it was shown that resistance was most likely inherited as an autosomal dominant gene. We have recently begun a series of experiments designed to establish linkage of this gene with known enzymatic and antigenic polymorphisms. Using mice from the (SJL/J x C57BL/6J)$_{F1}$ x SJL/J backcross we have been systematically comparing the segregation of resistance to EMC-induced diabetes mellitus with a panel of known polymorphic traits also segregating in this cross. To date, we have determined that there is no apparent linkage with chromosomes 7 (albino and pink-eyed dilution) or 17 (H-2 complex). Similar results have been obtained with Coxsackie B4-induced diabetes mellitus in animals of the (SWR/J x C57BL/6J)$_{F1}$ x SWR/J backcross.

In collaboration with Dr. B. A. Taylor of the Jackson Laboratory, we are also testing mice of B x D recombinant inbred strains for susceptibility to EMC-induced diabetes. These strains had randomly segregated DBA/2J and C57BL/6J genes before they were inbred. By comparing the susceptibilities of these strains with the profiles of the known DBA/2J and C57BL/6J markers that they possess, we hope to obtain additional information about the chromosomal location of the gene in question.

Effects of viral infection on cell surface receptors. Using a radioreceptor assay for insulin binding, it was shown that the binding of insulin to the receptors on the surfaces of human cells (WISH cells) was reduced early after infection with herpes simplex virus type 1 (HSV-1). Binding of radioactive insulin was reduced 50-60% in infected cells relative to control cells, and the magnitude of the reduction in binding was dependent upon the multiplicity of infection. UV-irradiated HSV-1 failed to change the kinetics of insulin binding, and virus-induced enzymes and interferon did not appear to contribute to the decrease in the number of insulin receptors. The half-life of the insulin receptor on WISH cells was measured to be greater than 24 hours, therefore the reduction could not be explained by an inhibition of receptor synthesis and normal turnover.

Similar results were obtained following infection of WISH cells with vesicular stomatitis virus, however, other viruses such as EMC (which does not induce cell surface antigens) and measles and Sindbis (which induce surface antigens late in the infection cycle) did not alter the number of insulin receptors.

These results suggest that early synthesis of virus-induced surface antigens, rather than inhibition of macromolecular synthesis, are most likely responsible for the decreased insulin binding. The mechanism by which this occurs is unknown and is being studied at present.

Significance to Biomedical Research

To a great extent, receptors are key intermediates in the response of cells to hormones, the infection of host cells by viruses, and the immune response. In man as well as in animal models, many debilitating diseases have been shown to be related to decreased number or function of receptors. Others are associated with known cell surface antigens, and others are caused by agents which require an appropriate surface receptor. In some cases, the relationship between the receptor and the disease has been characterized (e.g., adult obesity and insulin resistance). However, we are still left with a large degree of ignorance concerning the role of the cell surface in determining susceptibility to disease. By the appropriate study of selected membrane receptors, the relationship of receptor function and/or dysfunction to disease may be illuminated.

Course of Future Studies

Our immediate plans are to characterize the human and murine receptors for picornaviruses with respect to their physical-chemical properties and how these properties are related to differences in susceptibility in the cell tissues and individuals. At present, we are attempting to assay for picornavirus receptors in partially purified membrane fractions of tissue from which single cell preparations are not practical. Since tissue susceptibilities appear to differ within and among mouse strains, we hope to be able to define the reasons for these tissue tropisms. With a combination of biochemical, biological and genetic approaches, the nature of the receptors, the degree to which different viruses share receptors, and whether EMC receptors have other cellular functions will be studied.

The study of the influence of viral infection upon surface receptors will focus on defining the mechanism of the reduction in insulin receptor number by HSV-1 and VSV infection. In addition, we will attempt to determine whether other receptors such as those for acetylcholine, thyroid-stimulating hormone, and growth hormone are affected by viral infection *in vitro*.

ANNUAL REPORT OF THE
NEUROBIOLOGY AND ANESTHESIOLOGY BRANCH
NATIONAL INSTITUTE OF DENTAL RESEARCH

The Neurobiology and Anesthesiology Branch is concerned with the elucidation of basic mechanisms of oral-facial sensation with particular emphasis on pain sensation, and with the assessment and measurement of experimental and clinical pain in humans. Fundamental research on peripheral and central nervous system mechanisms of oral-facial sensation is conducted by the Neural Mechanisms Section. The primary objective of this research group is to correlate behavioral responses to noxious and non-noxious stimuli with neuronal function at various levels of the trigeminal system. A multidisciplinary approach is employed and includes the following research activities: (1) electrophysiological studies of the response of trigeminal brain stem and spinal cord neurons to noxious and innocuous thermal and mechanical stimuli; (2) behavioral and pharmacological studies involving pain and temperature discrimination in monkeys trained to escape potentially-noxious or noxious stimuli and to detect non-noxious stimuli applied to the face; (3) correlative behavioral and neural studies to determine the role of different peripheral and central neural populations in pain and temperature discrimination; and (4) psychophysical studies on the measurement and assessment of experimental and clinical pain.

Fundamental studies on neuronal structure and function are carried out by the Section on Neurocytology and Experimental Anatomy. This program is concerned primarily with neurocytological studies of the postnatal maturation and adult organization of the brain stem trigeminal nucleus in cat and monkey, utilizing light and electron microscopy and various tracer techniques. Correlative studies on neuronal structure and function are carried out in conjunction with investigators in the Neural Mechanisms Section. The Anesthesiology Section conducts clinical research on the use of intravenous sedative and anesthetic techniques to control anxiety and apprehension associated with dental procedures. Present activities include physiological, pharmacological and psychological studies of the effects of intravenous sedative drugs during and after oral surgery procedures.

Although the programs of the Branch are divided into these three Sections, there is considerable interactions between research groups. Correlative studies on neuronal structure and function are being carried out to elucidate in detail the circuitry of the upper layers of the dorsal horn of the medulla and spinal cord and their relationship to pain mechanisms. Studies of chronic pain in humans involve the use of behavioral and psychophysical techniques that also have been employed in correlative animal behavioral and physiological studies. The effects of intravenous sedative drugs are evaluated utilizing the improved psychophysical assessment methods developed by the pain measurement group. Finally, our previous animal research has provided the conceptual framework for present studies on endogenous pain-suppressing mechanisms.

In this last year, considerable progress has been made in developing a program for the sensory evaluation of chronic pain patients. In collaboration with local consultants, we now have a multidisciplinary team with expertise in neurology, neurosurgery, anesthesiology, psychiatry, psychology, pharmacology and oral surgery. This group evaluates patients admitted to the Clinical Center for a 2-3 week period. This detailed experimental and clinical work-up forms the baseline against which the efficacy of new pain control methods is evaluated.

After 42 years of government service, over 20 of them at the NIDR, Dr. Edward Driscoll, Chief of the Anesthesiology Section retired. Dr. Driscoll was one of the pioneer researchers in the field of dental anesthesiology and his research initiative and leadership have been critical for the development of the clinical program of the Branch. The Anesthesiology Section under his leadership has conducted unique studies on the use of different combinations of pharmacological agents useful in the relief of anxiety and apprehension associated with dental procedures.

Investigators in the Branch participated in a Consensus Development Conference on Pain, Discomfort and Humanitarian Care held at the NIH in February. This conference was held under the auspices of the Interagency Committee on New Therapies for Pain and Discomfort, composed of scientists and physicians from the NIH and other federal government agencies. A major goal of this group is to encourage research on the mechanisms and appropriate treatment of severe pain.

Other investigators in the Branch participated in a workshop at Cold Spring Harbor Laboratory on the neurobiology of pain. Major emphasis here was on the development of new immunocytochemical techniques for characterizing neuronal pathways involved in pain mechanisms. Finally, members of the Branch were invited speakers at symposia presented at the Society for Neuroscience, the American Pain Society, and the Association for Research on Nervous and Mental Diseases.

Anatomical Studies

These studies are aimed at delineating the synaptic circuitry of the caudal end of the trigeminal brain stem nuclear complex and its role in the processing of pain and temperature information. The caudal end of this nucleus called nucleus caudalis, or the dorsal horn of the medulla, is laminated and consists of an outer marginal layer (layer I), a middle substantia gelatinosal layer (layers IIa and IIb), and an inner magnocellular layer.

Recently we have shown that the superficial layers of trigeminal nucleus caudalis are organized similarly to the spinal cord dorsal horn. In light of this finding, we have now proceeded in both the trigeminal system and the spinal cord to elucidate the neural circuitry involved in spinal and brain stem pain mechanisms. The objectives of these studies during the past year were twofold. First, small caliber primary axons were filled with the protein marker, horseradish peroxidase (HRP). This marker can be visualized at light and ultrastructural levels and is used to determine the laminar distribution of these axons in the superficial layers of the spinal and medullary dorsal horns. Second, we have examined the synaptic connections of the two major interneurons in the substantia gelatinosa (SG) layer by combining HRP labeling with the intracellular recording method. This allows us to physiologically characterize these neurons and subsequently analyze their synaptic connections at light and electron microscopic levels.

Individual small caliber myelinated primary axons are found in SG with their major extensions in the rostracaudal axis of these layers. Each of these axons bears numerous axonal endings which synapse on two different kinds of dendrites; those with synaptic vesicles and those without synaptic vesicles. Our recent combined HRP and electrophysiology studies have shown that each of these dendrites is associated with a major type of interneuron found in the SG. One type, called stalked cell, has its cell body along the I/II border and gives rise to a cone-shaped dendritic arbor which traverses the SG. Its axon enters layer I. The second type, called islet cell, gives rise to extensive rostral and caudal dendritic arbors in SG. Its axonal arbor generates numerous branches within the confines of the dendritic arbor. Both of these cell types receive input from the small caliber primary endings terminating in SG. The islet cells contain clusters of vesicles in their dendritic spines which receive primary afferent input. Stalked cell dendrites which have synaptic contacts with primary axonal endings do not contain such vesicles.

Islet cell dendrites containing vesicles are presynaptic to several different neural processes. They form dendrodendritic synapses on non-vesicle containing dendrites, dendroaxonic synapses on primary axons, and dendroaxonic synapses on the shafts of unmyelinated axons. The primary axons and the two interneurons in SG are thought to function in the following manner. Axonal endings in SG activate stalked cells which are considered excitatory interneurons. Their axons enter layer I and synapse on second order projection neurons located there. The islet cell in SG functions as an inhibitory interneuron by inhibiting neural activity in the stalked cell via dendrodendritic synapses on stalked cells, dendroaxonic endings on primary endings, and axodendritic synapses on stalked cell dendrites.

During the past year, studies also were performed to identify the sources of input to the medullary dorsal horn from higher brain centers. These inputs appear to play a role in the modulation of sensory information. Descending projections to the caudal medulla arise from neurons in the raphe nuclei, the nucleus locus coeruleus, the reticular formation, and the periaqueductal grey matter. Some of these endings are likely to be sources of serotonergic and noradrenergic axonal endings in layers I and II of the superficial dorsal horn. These descending inputs play a role in descending control mechanisms and the production of analgesia.

Other studies have used autoradiographic techniques at light and electronmicroscope levels to characterize the morphology of serotonin-containing and norepinephrine-containing axons in the medullary dorsal horn. Electron microscopic analysis of axonal endings labeled with tritiated serotonin demonstrates two categories of morphologically distinguishable endings: dome-shaped endings which form single synapses and scalloped endings which form multiple synapses. These endings synapse primarily on small caliber dendritic shafts and spines. Dome-shaped endings were most common and three different types were found in layers I and/or II. One type of scalloped ending was present in layer I while layer III contained three types. Dome-shaped and scalloped-shaped norepinephrine endings also are present in these layers. They can be further subdivided into five types. The presence of these serotonergic and noradrenergic endings in layers I and II suggest that they have access to both the projection neurons in layer I and the interneurons in the SG or layer II. It also has been observed that both types of endings synapse on the same dendrite, suggesting that both monaminergic systems play a role in the modulation of the output of single neurons.

Correlative Anatomical and Physiological Studies

Previous anatomical studies in our laboratory have provided a detailed analysis of the morphology of neurons in the upper layers of the dorsal horn that presumably participate in pain mechanisms. However, the function of the various types of projection neurons and interneurons identified in these anatomical studies is only poorly understood. In recent studies we have attempted to correlate structure and function using an intracellular labeling technique. Neurons in layers I and II have been functionally characterized and then intracellularly stained with horseradish peroxidase.

Our results confirm that there are two common morphological types of SG neurons, stalked cells and islet cells. The stalked cell's physiological responses are of two general types. Nociceptive-specific neurons respond only to noxious stimuli. Wide-dynamic-range neurons respond to both innocuous mechanical and to noxious stimuli. These responses are very similar to those of projection neurons in layer I. This finding supports the hypothesis that stalked cells are excitatory interneurons that relay primary afferent input to layer I neurons.

There appear to be two types of islet cells. Islet cells in the superficial part of SG (layer IIa) have dendritic arbors with rostrocaudal expanses of 400-900 μm. Nearly all of the dendrites are contained within layer IIa. These cells have either nociceptive-specific or wide-dynamic-range responses. Islet cells with dendrites confined to the deep part of SG (layer IIb) have rostrocaudal extensions of 1200-1400 μm. Their physiological responses indicate that they are innervated only by low threshold mechanoreceptive primary afferents. These differences between layer IIa and IIb islet cells suggest that each type may be involved in separate inhibitory processes.

This study should provide long needed knowledge on the role of the substantia gelatinosa (layers IIa and IIb of the dorsal horn) in pain mechanisms as well as adding to our understanding of the function of short interneuronal circuits in the brain.

Physiological and Behavioral Studies

In previous studies we determined the peripheral nerve fiber groups responsible for the ability of the monkey to detect innocuous thermal stimuli and to escape noxious thermal stimuli applied to the face. These correlated behavioral and neurophysiological studies formed the foundation for present and future studies of central nervous system coding of pain and temperature sensation.

Where do these different peripheral fiber populations project in the central nervous system and what are the properties of central neurons involved in the monkey's ability to detect non-noxious and noxious thermal stimuli. We began to answer these questions by examining the response properties of neurons in trigeminal nucleus caudalis in the brain stem in anesthetized monkeys. Many of these neurons are part of a pathway that projects directly to the thalamus and is exclusive of other components of the trigeminal brain stem nucleus. This analysis revealed two general classes of trigeminothalamic neurons that convey information related to pain. Current studies extend these observations to the awake, behaving monkey. These studies allow detailed analyses of how the brain codes various properties of nociceptive input. In contrast to the relatively static and passive input-output relationships attending experiments on anesthetized preparations, studies of awake behaving monkeys provide the opportunity to examine variables such as attention, response appropriateness, expectation and stimulus salience, and the role they play in nociception.

Monkeys were trained to detect the termination of innocuous heat stimuli and the onset of noxious heat stimuli (45°-49°C). In a second task, the same monkeys detected the onset of a light stimulus while behaviorally nonrelevant thermal stimuli were presented. Neuronal activity in the dorsal horn of the medulla has been correlated with a number of behavioral events such as panel press, temperature onset and termination, and panel release. Two classes of neurons were studied. Nociceptive neurons exhibited responses correlated with the onset and termination of thermal stimuli. In both behavioral tasks, thresholds often were below 45°C, and stimulus-response functions were positively-accelerating in the noxious heat range. However, the sensitivity of these neurons was contingent upon the behavioral situation. Responses to behaviorally non-relevant thermal stimuli presented during the second task (light task) were reduced in magnitude as compared to stimuli presented during the thermal task. Increasing vigilance demands by reducing the intensity of the light further reduced the amplitude of heat-evoked neuronal activity. Finally, a warming signal predicting the onset of a noxious heat stimulus (47° or 49°C) decreased heat-evoked activity during the thermal task, as compared to unsignaled trials. A second class of neuron exhibited maximum activity during the execution of task-related behaviors. Such task-related neurons exhibited a maintained discharge during the panel press period. Discharge rate was independent of stimulus intensity or modality, since the maintained discharge was present in both tasks and occurred in the absence of thermal stimulation. A burst of neuronal activity occurred in both tasks prior to panel release. The burst was absent if the monkey failed to detect the cue or released inappropriately. Burst magnitude was independent of the intensity of the relevant cue and its latency was more highly correlated with panel release than with stimulus termination. The burst discharge occurred on experimenter-initiated trials in which monkey hand movements were not present. In addition, the burst discharge could be temporally dissociated from lip movements made in preparation of liquid reinforcement. Thus, task-related responses at panel press and panel release were independent of physical characteristics of the stimulus and could be dissociated from motor preparatory behavior. Nociceptive neurons often exhibited task-related responses but of less magnitude than their responses to noxious thermal stimulation. These data suggest that nociceptive neurons provide information about the intensity of noxious heat stimuli and can be modulated by behavioral contingencies. These functional properties demonstrate the importance of modulatory influences on medullary dorsal horn neurons in alert behaving animals. Finally, the task-related responses of these neurons indicate that variables such as stimulus relevance, attentional processes, response appropriateness, and reinforcement contingencies alter neuronal activity at the brain stem level. Such responses appear to be critical in signalling the chain of neural events from stimulus perception to motor response that are necessary for the execution of goal directed behaviors.

We have employed the same behavioral thermal task in pharmacological studies in awake monkey to assess the relative effects of morphine on the sensory discriminative and affective dimensions of responses to noxious thermal stimuli. Our results indicate that morphine attenuates the monkey's ability to discriminate noxious and innocuous thermal stimuli independent of its effect on the aversive quality of the stimulus. This result parallels results in human experiments described below: narcotic analgesic drugs appear to alter the sensory discriminative aspect of the pain experience apart from their effect on its affective component. The results in the animal studies also demonstrate that our behavioral model may be useful in the evaluation of new pain control agents.

Human Psychophysical and Clinical Studies

The clinical program of the branch was established to take advantage of new knowledge gained in the laboratory and to apply it to the clinical situation. This program has been expanded this year to include new studies of chronic oral-facial pain conditions.

A major area of study in this program involves the development of psychophysical measurement techniques for experimental and clinical pain, and the use of validated techniques to evaluate pharmacological and non-pharmacological pain control agents. The reliable, objective, and valid verbal descriptor scales developed in this project have been used to assess postsurgical pain following third molar extractions in nonsedated patients. Pain was assessed before and after the intravenous administration of either a narcotic antagonist (naloxone) or a placebo, or after no treatment. Verbal descriptor questionnaires showed that postsurgical pain following placebo was significantly reduced in comparison to the pain experienced after either naloxone or no treatment. The demonstration that naloxone results in increased pain sensitivity as compared to placebo supports the hypothesis that it reverses the action of endogenous opioid compounds produced as a consequence of the surgery.

An additional study used verbal descriptor measures and experimental pain matching techniques to assess pain in patients suffering from chronic oral-facial pain syndromes. These patients were able to scale their clinical pain as readily and reliably as pain produced by experimental tooth pulp stimulation and they readily matched the sensory intensity of these qualitatively different types of pain. These results show that a combined matching-scaling paradigm can provide consistent results in chronic pain patients.

Studies recently have been initiated to assess the mechanisms and relative efficacy of brain stimulation and narcotic analgesics for the control of chronic intractable human pain. These studies will compare brain stimulation to conventional narcotic administration in terms of (1) magnitude of analgesia; (2) mechanisms of analgesia; (3) adverse side effects; and (4) tolerance.

Other experiments continue to evaluate the quality of sensations elicited from the tooth pulp and the underlying neural mechanisms responsible for these sensations. All subjects report sensory detection thresholds that are significantly lower than pain thresholds at all frequencies of stimulation used. These findings suggest that non-nociceptive input may originate from the tooth pulp, previously presumed to be a pure pain source. This interpretation is supported by other findings. Subjects are able to scale the intensities of these non-pain sensations. In addition, the electromyographic silent period in masseter muscle produced by tooth pulp stimulation can be dissociated from the perceived pain sensations by the administration of a narcotic analgesic or by conditioning electrical stimulation to the tooth pulp. These manipulations alter pain sensations but have no effect on the masseter inhibitory or silent period. Both effects on pain sensations can be reversed by naloxone administration. It appears likely that reflex activity not associated with pain sensations may arise from tooth pulp and utilize distinct central neural pathways. Activity in the pain pathway is modulated by descending opiate-like pathways, whereas the reflex pathways are not.

Our studies on the effectiveness of intravenous sedation techniques in the past on dental patients have concentrated on amnesia effects and recovery of sensory and motor functions. In the past year, our efforts have been towards an evaluation of physiological responses, such as cardiac function, blood pressure, and oxygen saturation. The physiological responses are monitored non-invasively to avoid introducing further anxiety and morbidity which may be associated with invasive techniques. Our results indicate that drug combinations that result in greater degrees of sedation are accompanied by measurable impairment in cardiovascular and respiratory hemostasis. These combinations have no clear advantage to the patient in terms of anxiety-relief or amnesia. Our future goal is to continue to evaluate physiological and psychological function in this experimental model in order to identify pharmacological as well as non-pharmacological techniques that produce anxiety relief and a reduction in patient apprehension with a minimal potential for clinical toxicity.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 DE 00132-05 NA

PERIOD COVERED
October 1, 1978 to September 30, 1979 CT 0060102

TITLE OF PROJECT (80 characters or less)
Psychophysiological Assessment of Intravenous Sedation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Dionne, Raymond A.	Staff Fellow	NIDR NA
	Driscoll, Edward J.	Chief, Anesthesiology Sec	NIDR NA
OTHER:	Amato, Peggy R.	Clinical Nurse	NIDR NA
	Butler, Donald P.	Oral Surg-Anesthesiologist	NIDR NA
	Clark, Barbara Ann	Clinical Nurse	NIDR NA
	Streko, Thomas	Staff Dentist	NIDR NA
	Sweet, James B.	Sr. Dental Surgeon	NIDR IR

COOPERATING UNITS (if any)

LAB/BRANCH
Neurobiology and Anesthesiology Branch

SECTION
Anesthesiology Section

INSTITUTE AND LOCATION
NIDR, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.30	1.25	1.05

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this project is to systematically assess the physiological responses to drug combinations used to alleviate anxiety in dental outpatients. Cardiovascular and respiratory functions are monitored non-invasively by means of an impedance cardiograph, earpiece oximeter and automatic blood pressure recorder. Results of the project indicate that the combination of diazepam-fentanyl and methohexitol causes a decrease in stroke volume which is accompanied by a reflex tachycardia. This combination also results in a decrease in arterial oxygen saturation. The use of diazepam alone and diazepam plus fentanyl does not result in any detectable cardiovascular or respiratory effects. The methodology employed in this study will be used to assess other intravenous, inhalational, and oral agents which can be useful for alleviating pain and anxiety in dental outpatients.

PHS-6040
(Rev. 10-76)

1. Project Description:

Objectives: The purpose of the project is to assess the physiological response to drug combinations commonly used in dental outpatients to relieve anxiety. These agents are used in moderately to highly anxious patients undergoing routine dental procedures and in patients undergoing extensive procedures such as the removal of third molars and periodontal surgery. Despite their relatively widespread use in clinical practice, no systematic assessment has been made of the various agents and combinations. By combining the results of this assessment with the results of previous studies which quantified the various parameters of therapeutic efficacy such as amnesia, sedation and anxiety relief, it is possible to quantify the relationship between efficacy and clinical toxicity, i.e., the therapeutic ratio. This information provides an objective, rational basis for selecting the optimal drug or combination for the various therapeutic situations in which they are used. In addition, the therapeutic ratio provides an objective method for assessing new agents proposed for use in alleviating anxiety in dental outpatients.

Methods Employed: Potential patients, 18 years or older of either sex, are screened by members of the NIDR Dental Clinic to verify the need for extraction of third molar teeth, the surgical procedures elected for use in this study. After validation of this surgical need, a complete history and physical examination (including appropriate laboratory studies) is performed by qualified NIDR Staff.

The response to four different drug combinations and to placebo are studied. Patients are randomly allocated to the various treatment groups. These treatments are: 1) diazepam 15 mg, methohexitol 10 to 40 mg; 2) diazepam 12.5 mg, fentanyl 0.10 mg and methohexitol 10 to 40 mg; 3) fentanyl 0.001 mg/kg, diazepam 0.22 mg/kg; 4) diazepam to clinical endpoint; 5) saline placebo. All drugs are administered intravenously and all patients receive local anesthesia. A within subject design is used to compare treatment #1 to treatment #2 and to compare treatment #4 to placebo. All groups are compared to each other and to placebo by appropriate between subjects analysis.

The physiological response is monitored non-invasively to avoid introducing further anxiety and morbidity which can be associated with invasive techniques. Oxygen saturation is recorded via an earpiece oximeter, blood pressure via an automatic blood pressure device and heart rate, stroke volume and cardiac output with an impedance cardiograph. Preoperative baseline readings are taken every 5 minutes, intraoperative readings are taken every minute and post-operative readings every 5 minutes. This permits comparison of maximum drug effects to baseline values and comparison of maximal effects of the different treatments. The time of recovery to normal baseline is also monitored.

Major Findings: No substantial decrease from baseline cardiac output was seen following any of the four treatments. The group receiving the diazepam-fentanyl-methohexitol combination showed a transient decrease in stroke volume accompanied by a reflex tachycardia. This was seen to a lesser extent in the diazepam-methohexitol group. No significant changes were seen following diazepam-fentanyl or diazepam alone. The placebo group showed a marked increase in heart rate and cardiac output during surgery. Oxygen saturation was also significantly depressed following the combination of diazepam, fentanyl and methohexitol. The mean arterial blood pressure was not affected deleteriously by any of the treatments.

These results indicate that a widely used sedative combination, diazepam-fentanyl-methohexitol, is accompanied by an alteration of cardiovascular and respiratory homostasis. This is true to a lesser extent for the combination of diazepam and methohexitol. Diazepam alone and diazepam plus fentanyl do not result in any detectable physiological impairment. The justification for employing combinations that include fentanyl and methohexitol is to enhance amnesia, sedation and patient cooperation. Previous work by this section and other investigators suggest that decreasing the amount of diazepam to allow the use of these CNS depressants results in a decrease in amnesia, rather than an increase. Previous work has shown a marginal advantage in terms of sedation, the patient's external manifestation of tranquility, and patient cooperation for the combination of diazepam, fentanyl and methohexitol but evidence also suggests that all of the combination tested result in clinically acceptable levels of sedation and patient cooperation. Finally, it has been demonstrated that patient's cannot differentiate between these combinations in terms of anxiety relief, a major determinent of therapeutic efficacy. This body of work suggests that the use of combinations of drugs such as diazepam-methohexitol and diazepam-fentanyl-methohexitol are resulting in detectable physiologic impairment without any substantial increase in therapeutic effect.

Significance to Biomedical Research and Program of the Institute: The ultimate goal of the Institute is to develop information and technologies which result in an improvement in dental care. The delivery of any dental care is contingent upon regular professional visits. Although it has not been scientifically demonstrated, it is generally accepted that a certain percentage of the population avoids or postpones care due to anxiety about the pain that they may experience. Intravenous sedation is a technique for overcoming the anxiety acutely and, theoretically, results in a decrease in anxiety towards dentistry by allowing for a tranquil non-painful experience. By studying the physiological and psychological responses, we have been able to identify the drug combinations that are effective for these goals with a minimal potential for clinical toxicity. The publication of this information will add to the body of knowledge needed to lead to general acceptance and use of intravenous sedation. The more widespread use of intravenous sedation will ultimately result in enhanced delivery of dental care and improved dental health.

Proposed Course: Future studies are aimed at assessing other modalities for pain and anxiety control in dental outpatients. These include oral premedication with non-steroidal anti-inflammatory analgesics such as flurbiprofen, and the use of long-acting local anesthetics such as bupivacaine and etidocaine. In addition, the efficacy of intravenous diazepam plus nitrous oxide will be compared to diazepam alone. The overall goal of these three projects will be to develop methods for pain and anxiety control in dental outpatients that do not require special training or result in prolonged periods for recovery or alteration in physiological homeostastis.

2. Publications:

Gelfman, S.S., Gracely, R.H., Driscoll, E.J., Wirdzek, P.R., Butler, D.P. and Sweet, J.B.: Comparison of recovery tests after intravenous sedation with diazepam-methohexitol and diazepam-methohexitol and fentanyl. J. Oral Surg., 37:391-397, 1979.

Driscoll, E.J. and Gelfman, S.S.: Thrombophlebitis: Its Incidence and Natural History. J. Oral Surg., 37: In Press, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 DE 00276-01 NA

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Narcotic and Brain Stimulation Analgesia on Human Chronic and Experimental Pain

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Gracely, Richard H.	Research Psychologist	NIDR NA
OTHER:	Dubner, Ronald	Chief, NAB	NIDR NA
	Dionne, Raymond A.	Staff Fellow	NIDR NA
	Hoffert, Marvin J.	Senior Staff Fellow	NIDR NA
	Amato, Peggy R.	Clinical Nurse	NIDR NA
	Lees, David E.	Deputy Chief	CC ANES

COOPERATING UNITS (if any)
Dr. Richard Greenberg
Division of Neurosurgery
Virginia Commonwealth University, Richmond, Virginia

LAB/BRANCH
Neurobiology and Anesthesiology Branch

SECTION
Anesthesiology Section

INSTITUTE AND LOCATION
NIDR, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.90	0.75	1.15

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)
The purposes of the study are (1) Assess the effectiveness of chronic electrical stimulation of midbrain sites for the relief of chronic pain in humans; (2) Evaluate the efficacy and mechanisms of traditional narcotic analgesia and compare these to chronic electrical stimulation of midbrain sites; (3) Validate experimental models of pain and their potential diagnostic use in chronic pain patients; and (4) Determine and compare the impact of both traditional narcotic and chronic electrical stimulation therapies on the functional, intellectual and emotional well being of these patients. Participants in this study will be (1) chronic pain patients receiving surgically implanted stimulating electrodes for pain control; (2) chronic pain patients maintained on traditional narcotic analgesics who will not receive implanted stimulating electrodes; and (3) healthy normal volunteers. The effects of chronic brain stimulation in surgical patients will be compared to the effects of narcotics previously administered to patients and to effects of narcotic regimes in nonsurgical chronic pain patients. In addition, the effects of narcotics on perceptual and neural mechanisms of experimentally induced pain and on normal psychological functioning will be compared between chronic patients and normal volunteers free of chronic pain.

1. Project Description

Objectives: These studies will assess both the mechanisms and relative efficacy of brain stimulation and narcotic analgesics for the control of chronic, intractable human pain. Stimulation of midbrain sites is a recent analgesic technique performed by a small number of neurosurgeons. The present studies address several issues pertient to the use of this method. How does brain stimulation compare to conventional narcotic administration in terms of: (1) magnitude of analgesia, (2) mechanisms of analgesia, (3) adverse side effects, and (4) tolerance.

Methods Employed: Patients scheduled to receive implants are admitted to the NIH Clinical Center before and after surgery for extensive testing including neurological workups, psychiatric and psychological evaluation, appropriate laboratory tests, clinical pain questionnaires, responses to noxious tooth pulp and heat stimulation, and tests of cognitive and psychomotor functioning. Most patients are assessed before and after administration of analgesics, placebos, narcotic antagonists or brain stimulation. Patients not receiving implanted electrodes and normal volunteers are assessed in a similar manner.

Major Findings: Eight patients have been assessed to date. Five have received electrodes, one was rejected and two are scheduled for implanation. Of the five receiving electrodes, three had little pain relief within one week of surgery and the electrodes were removed. Two of these had required large doses of narcotic medication for pain relief and had developed considerable tolerance to these medications, 15 mg morphine did not alter appreciably either experimental or clinical pain measures. The third electrode removal was from a patient with atypical facial pain who was not narcotic tolerant. Administration of morphine, however, did not relieve her clinical pain.

Of the two patients with electrodes implanted for more than one week postsurgery, one had satisfactory pain relief for 7 months before the electrode was removed because of complaints about scalp irritation over the electrode site. The other has had the electrode for a short time and has not been reevaluated at NIH.

Significance to the Biomedical Research Program of the Institute: The present results provide additional evidence that the analgesia produced by brain stimulation is cross-tolerant to analgesia produced by narcotic administration and that brain stimulation cannot be utilized in narcotic tolerant patients without at least a suitable weaning from narcotic medication. The negative results with atypical facial pain suggest that this syndrome may not respond to this procedure. The lack of pain relief by conventional narcotic analgesics in this patient supports the hypothesis that responsitivity to narcotic analgesics may predict the efficacy of brain stimulation analgesia.

Proposed Course: The assessment of patients scheduled to receive electrode implants will continue with an anticipated load of 20 patients yearly. During postoperative admissions, the efficacy of analgesia produced by brain stimulation will be compared to that originally produced by narcotic analgesics. An additional group of chronic pain patients not scheduled to receive implanted electrodes also will be assessed to control for order effects. The experimental procedures will be used also to assess the analgesic, cognitive and motoric effects of narcotics in normal volunteers free of pain and narcotic tolerance. These procedures will generate data on the neuronal and cognitive effects of narcotic analgesia and narcotic tolerance.

2. _Publications_:

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 DE 00031-11 NA

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Design and Computer Interfacing of Neurophysiologic Instrumentation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Brown, Frederick J.	Electronic Engineer (Instru)	NIDR NA
	Medlin, Terry P.	Supv. Computer Specialist	NIDR OD

COOPERATING UNITS (if any)

LAB/BRANCH
Neurobiology and Anesthesiology Branch

SECTION
Neural Mechanisms Section

INSTITUTE AND LOCATION
NIDR, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 1.00	PROFESSIONAL: 1.00	OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This work involves the development of suitable electronic and electromechanical instrumentation to be used in neurophysiological, physiological and behavioral research. It involves the adaptation and interfacing of these and other instruments to a laboratory or multipurpose computer installation.

1. Project Description

a) A DEC 11/34 computer has been interfaced to equipment in one laboratory through an INTEL SBC-80/10 computer. The 11/34 interacts with scientists in the laboratory while controlling a behavioral experiment on monkeys. Collection of the behavioral data and control of the equipment is aided by the SBC-80/10. Neurophysiological data from the monkey are presently recorded on analog magnetic tape for later computer processing.

The 11/34 computer has also been interfaced to a data entry facility. Here nerve cell and EMG data previously recorded on analog magnetic tape are entered into the 11/34 through an INTEL MCS-85 microcomputer. The MCS-85 allows accurate timing of the neurophysiological and behavioral events while leaving the 11/34 relatively free to process data or run other experiments.

b) A pair of high gain, high impedance, low noise, differential amplifiers have been designed and built to facilitate the recording of electrical signals from awake monkeys. The amplifiers are used to monitor single cell activity of neurons in the brain or EMG muscle activity.

2. Publications:

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF** **INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 DE 00040-10 NA

PERIOD COVERED

October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Activity of Trigeminal Afferent Fibers in Response to Noxious and Innocuous Stimuli

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Dubner, Ronald	Chief, NAB	NIDR NA
	Seltzer, Zeev	Visiting Fellow	NIDR NA
	Yokota, Toshikatsu	Visiting Scientist	NIDR NA

COOPERATING UNITS (if any)

LAB/BRANCH

Neurobiology and Anesthesiology Branch

SECTION

Neural Mechanisms Section

INSTITUTE AND LOCATION

NIDR, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 1.65	PROFESSIONAL: 1.30	OTHER: 0.35

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Under barbiturate anesthesia, microelectrode recordings from the trigeminal ganglion of monkey are performed and electrical activity is recorded and stored on magnetic tape. Thermal and noxious heat stimuli are delivered with a precisely-controlled thermode which has a temperature range of 20°C to 60°C and can produce a temperature change of 10°C at the thermode-skin junction within one second. Data are analyzed with the aid of an on-line real-time computer system. In related experiments, intracellular recordings are made from primary afferent neurons in trigeminal dorsal root ganglia, dorsal root fibers, or the spinal trigeminal tract, in response to innocuous and noxious thermal and mechanical stimuli. After functional characterization, horseradish peroxidase is iontophoresed into the cell or fiber. This intracellular label is then located in histological material and the central terminal projections of each axon determined.

1. Project Description

Objectives: In this study we have examined the response properties of peripheral nerve fibers that innervate the face and body skin. We have attempted to characterize these neurons based on their response to innocuous and noxious levels of mechanical and thermal stimuli applied to receptive fields. One major aim is to correlate the activity of these peripheral neuronal populations with behavioral observations in monkeys trained to detect or escape thermal stimuli applied to the same region of their face. Another aim is to define the peripheral input systems that are capable of coding various aspects of sensation and to determine what transformations, if any, take place during central coding. A third aim is to correlate the physiological properties of these fibers with their terminal projections in different layers of the trigeminal brain stem nucleus and spinal dorsal horn.

Methods Employed: Under barbiturate or N_2O/O_2 anesthesia, microelectrode recordings are made from the trigeminal ganglion after hemispherectomy. With glass-coated tungsten microelectrodes, we are able to isolate single units including those innervated by unmyelinated (C) fibers. Thermal and noxious heat stimuli are delivered with a precisely-controlled thermode. Mechanical stimuli consist of hand-held probes and von Frey filaments. Data are recorded on magnetic tape and analyzed with the aid of an on-line real-time computer system. In the intracellular studies in the spinal cord, individual fibers are filled with horseradish peroxidase and central terminal projections later are examined with light and electron microscopy.

Major Findings: In previous experiments we have reported on the responses of A delta heat nociceptors and A beta high-threshold mechanoreceptors to warming and noxious heat. These data indicated that A delta heat nociceptive afferents are the only myelinated afferents that respond reliably to noxious heat. Correlation of their heat responses with escape latencies in monkey indicates that activity in this neural population is sufficient to account for fast escape latencies occurring at 49° and 51°C. Present experiments involve intracellular recordings from individual axons in the dorsal root entry zone. Successfully impaled axons are filled iontophoretically with horseradish peroxidase and central terminals later are examined at the light and electron-microscope level. Preliminary results indicate that different functional types of primary afferents project to different layers in the spinal cord dorsal horn.

Significance to Biomedical Research and Program of the Institute: These studies shed light on the way in which the brain processes information related to the presence of tissue-threatening or tissue-damaging stimuli in the environment. By defining the parameters of stimulation necessary to activate specific classes of nociceptors, and comparing them with psychophysical measurements using similar parameters, we can ascertain their role in pain sensation. Transformation or processing of activity from each nociceptive class then can be evaluated at different levels of the neuraxis. In addition, the animal behavioral model which has been developed in this laboratory, in combination with electrophysiological studies, should be useful in the development and testing of new and improved methods of pain control.

Proposed Course: We have initiated studies which correlate functional properties of primary afferents with the morphology and location of their terminal axonal arbors in the brain stem, utilizing intracellular tracer techniques.

2. Publications:

Dubner, R., Sessle, B.J. and Storey, A.T.: The Neural Basis of Oral and Facial Function. Plenum Press, New York, 1978, 483 pp.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 DE 00133-05 NA

PERIOD COVERED
October 1, 1978 to September 30, 1979 CT 0060101

TITLE OF PROJECT (80 characters or less)

Assessment of experimental and clinical pain

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Gracely, Richard H.	Research Psychologist	NIDR NA
OTHER:	Dionne, Raymond A.	Staff Fellow	NIDR NA
	Dubner, Ronald	Chief, NAB	NIDR NA
	Sweet, James B.	Sr Dental Surgeon	NIDR IR
	Heft, Marc W.	Clinical Assoc (Dentistry)	NIDR IR
	Streko, Thomas	Staff Dentist	NIDR NA
	Wolskee, Patricia J.	Psychologist	NIDR NA

COOPERATING UNITS (if any)

LAB/BRANCH
Neurobiology and Anesthesiology Branch

SECTION
Neural Mechanisms Section

INSTITUTE AND LOCATION
NIDR, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.70	1.60	2.10

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The objectives of this project are (1) to assess psychophysical methods of experimental pain measurement, i.e., magnitude estimation, category scaling, and cross-modality matching. Pain will be experimentally induced by electrocutaneous, electric tooth pulp, and mechanical heat stimulation; (2) to assess clinical pain measures, such as pain questionnaires and sensory matching methods, in a dental setting; (3) to determine the validity of experimental pain models by comparison of experimental and clinical pain responses; and (4) to evaluate known pharmacological and non-pharmacological pain-control agents.

PHS-6040
(Rev. 10-76)

1. Project Description

Objectives: The purpose of these studies is to develop psychophysical measurement techniques for experimental and clinical pain, and to use validated techniques to evaluate pharmacological and non-pharmacological pain control agents. Assessment techniques must attempt to independently measure the sensory and motivational dimensions of the pain experience. The sensory dimension is generally assumed to be associated with the discriminatory aspects of pain. The motivational or unpleasantness dimension is generally assumed to be a complex perceptual cognitive component influenced by psychological factors. There is evidence that these two factors may be distinguished at a physiological level and evidence which suggests that pain control agents can act differentially on these two pain components.

Methods Employed: Experimental pain is produced by electrical stimulation of skin and teeth, by heat applied to skin and by noxious cold stimulation of exposed dentin. The sensory intensity and unpleasantness associated with these stimuli are assessed by verbal descriptor scales and cross-modality matching techniques. Clinical pain is being assessed by verbal scaling, by sensory matches to experimental pain stimuli, and by questionnaire. The verbal pain descriptors have been objectively and reliably quantified in previous studies.

Major Findings: The reliable, objective and valid verbal descriptor scales developed in this project have been used to assess postsurgical pain following third molar extractions in nonsedated patients. Pain was assessed before and after the intravenous administration of either a narcotic antagonist (naloxone) or a placebo, or after no treatment. Verbal descriptor questionnaires showed that postsurgical pain following placebo was significantly reduced in comparison to the pain experienced after either naloxone or no treatment. Commonly used visual analog pain scales showed few significant effects. The experiments concerned with the assessment of narcotic and brain stimulation produced analgesia in chronic pain patients are described in a new NIRP, "Effects of Narcotic and Brain Stimulation Analgesia on Human Chronic and Experimental Pain".

An additional study used verbal descriptor measures and experimental pain matching techniques to assess pain in patients suffering from chronic oro-facial pain syndromes. A correlational analysis showed that selected experimental measures correlated with clinical measures of muscle tenderness in patients with Myofascial Pain Dysfunction Syndrome. The experimental measures also showed that these patients can scale their clinical pain as readily and reliably as pain produced by experimental tooth pulp stimulation and that they can reliably match the sensory intensity of these qualitatively different pains.

Results of additional studies of Myofascial Pain Dysfunction syndrome are presented in two additional NIRPs, "Sensations Produced by Tooth

Pulp Stimulation" and "Masseteric Exteroceptive Reflex and Sensory Responses in MPD Patients". A study of pain trigger points in trigeminal neuralgia showed that (1) oro-facial trigger points can vary over time in the same individual; moving between intra and extra-oral sites such as the gingiva, teeth, facial skin and facial hair. These studies also showed that painful paroxsym could be elicited by summation of repeated weak tactile stimulation and that paroxsyms are followed by a refractory period of several minutes during which no paroxsym can be elicited by any level of stimulation intensity or frequency.

A final study examined the variability of sensitivity to noxious thermal stimulation over the volar forearm within individuals. Pain thresholds are determined by a modified staircase method for each of 8 spots. Sensitivity varied nonsytematically over these spots.

Significance to Biomedical Research and Program of the Institute: The demonstration of increased pain sensitivity following administration of naloxone in nonsedated postsurgical patients provides evidence that naloxone increases pain by reversing endogenous opiate-like compounds produced as a consequence of surgery rather than by reversing sedative agents used during surgery.

The superior sensitivity of the verbal descriptor scales suggests that conventional analog pain scales are not optimal measures of pain experience. The negative results with analog scales in this study suggests that sedatives may have played a role in previous studies in which a single analog scale showed significant effects of naloxone in relation to placebo.

The study of patients with Myofascial Pain Dysfunction showed that verbal descriptor measures of the sensory intensity, unpleasantness and painfulness of the experience correlated with clinical impression of muscle tenderness. The results comparing verbal scales and matching methods indicate that chronic pain can be scaled as reliably as controlled experimental stimuli. These results also show that a combined matching-scaling paradigm can provide a measure of consistency for individual subjects.

Thermal stimulation is superior to other noxious stimulation methods because it is natural, it activates a restricted group of primary afferents, the stimulating range is fairly constant between individuals, and it permits assessment of neural mechanisms of suppression and summation. However, the temporal interaction of successive stimulation requires that certain studies use a number of stimulation sites within an individual. The interspot variability found in the present thermal experiments suggests that new paradigms that account for spatial sensitivity fluctuations should be developed before thermal methods are used to assess the effects of putative analgesic manipulations.

Proposed Course: Future studies on the role of endorphin modulation of postsurgical pain will determine if experimental pain measures can detect changes in endorphin levels produced by the anxiety, pain and trauma associated with oral surgery. Additional studies also will determine the role of endorphin release on placebo responsivity. The sensitivity of the verbal descriptor questionnaire used in the experiments will be optimized by standard multivariate techniques.

The studies of chronic oro-facial pain syndromes will continue with a longitudinal analysis of the effectiveness of conservative treatment techniques for Myofascial Pain Dysfunction Syndrome. These patients will also rate the sensory intensity or unpleasantness of experimental tooth pulp stimuli before and after the administration of either fentanyl or placebo to determine if the differential results observed previously with normal volunteers also occur with patients suffering from chronic pain.

The studies using noxious thermal stimulation will continue with the development of new behavioral and psychophysical paradigms that will assess the efficacy and mechanisms of narcotic analgesia. Studies using chronic pain patients will provide information about neural mechanisms of narcotic tolerance.

2. Publications:

Gracely, R.H., Dubner, R. and McGrath, P.A.: Narcotic Analgesia: Fentanyl Reduces the Intensity but not the Unpleasantness of Painful Tooth Pulp Stimulation. Science, 203:1201-1263, 1979.

Gracely, R.H.: Psychophysical Assessment of Human Pain. In press in: Bonica, J.J., Liebeskind, J.C. and Albe-Fessard, D., Advances in Pain Research and Therapy, Vol. 3, Raven Press, New York.

Gracely, R.H.: Pain Measurement in Man. In: Ng., Pain, Discomfort and Humanitarian Care. Proceedings of NIH Symposium, Feb. 1979, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 DE 00151-05 NA

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Trigeminothalamic Neurons Responsive to Innocuous and Noxious Stimuli

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	Hoffman, Donna	Postdoctoral Fellow	NIDR NA
OTHER:	Dubner, Ronald	Chief, NAB	NIDR NA
	Ziriax, John M.	Psychologist	NIDR NA
	Mueller, Roy	Psychologist	NIDR NA

COOPERATING UNITS (if any)

LAB/BRANCH
Neurobiology and Anesthesiology Branch

SECTION
Neural Mechanisms Section

INSTITUTE AND LOCATION
NIDR, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 3.65	PROFESSIONAL: 2.05	OTHER: 1.60

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project studied the responses of trigeminal nucleus caudalis neurons to noxious and innocuous mechanical and thermal stimuli in awake behaving rhesus monkeys. Monkeys were trained to detect the termination of innocuous heat stimuli and the onset of noxious heat stimuli. In a second task, the same monkeys detected the onset of a light stimulus while behaviorally nonrelevant thermal stimuli were presented. Nociceptive neurons exhibited thermal thresholds of 45°C or lower and monotonic stimulus-response functions to noxious stimuli presented during both tasks. Responses to thermal stimuli presented during the light task were reduced in magnitude when compared to responses during the thermal task. A second class of neuron exhibited its maximum activity during the execution of task-related behaviors. This activity was independent of stimulus characteristics and could be separated from motor activity. Nociceptive neurons also exhibited task-related activity, but it was of lesser magnitude than the responses to noxious thermal stimuli. The task-related responses of both classes of neurons may be associated with attentional processes involved in organizing goal-directed behaviors.

PHS-6040
(Rev. 10-76)

1. Project Description

Objectives: Previous work in this project employed anesthetized preparations to examine the response properties of nucleus caudalis and subjacent reticular formation neurons that are part of the trigeminal nuclear system involved in nociceptive responses by rhesus monkeys. The present studies represent an effort to evaluate the contribution of general anesthesia to the results of earlier work. Moreover they allow detailed analyses of the dynamic properties of the encoding of nociceptive input. In contrast to the relatively static and passive input-output relationships attending experiments on anesthetized preparations, studies of behaving monkeys provide the opportunity to examine the interactive properties of somatosensory systems. Variables such as attention, response appropriateness, expectation and stimulus salience can be studied, and their role in nociception evaluated.

Methods Employed: In the physiological single unit recording experiments, a microelectrode is stereotaxically introduced into the brainsteam trigeminal nucleus caudalis through a sealed chamber chronically fixed to the monkey's skull. During the experiment the monkeys are temporarily restrained. Otherwise the animals are kept unrestrained in their home cages.

In one behavioral task currently being used and reported on previously, monkeys are trained to detect the termination of innocuous heat pulses delivered via a feedback-controlled contact thermode (1 cm diam) applied to the upper hairy lip. Adapted skin temperature is 35^{o}C. A panel is illuminated to signal the beginning of each trial. Monkeys depress the lighted panel to initiate innocuous heat pulses (37^{o}, 39^{o}, 41^{o}, 43^{o}C) of randomly varying durations (2-8 sec). Panel releases of less than 2 sec (reaction times or RTs) after the termination of these stimuli are rewarded by water (0.4 cc). Additional heat pulses of a constant duration (20 sec) are presented quasi-randomly in which the temperature increase reaches the noxious range (45^{o}, 47^{o}, 49^{o}C). Release of the panel at the termination of noxious stimuli is not rewarded by water. However, early releases terminate heat pulses on these trials, thereby allowing the monkeys to escape noxious heat stimuli. Thus the task assesses RTs indicating the detection of the termination of innocuous heat stimuli or the discrimination of the onset of higher stimulus intensities shifting into the noxious range.

A second behavioral paradigm has been introduced to address the role of attentional processes as well as the effects of requiring the monkey to respond primarily to visual rather than thermal stimuli. In this task the water restricted monkey is trained to detect the onset of light stimuli. Correct detection is rewarded by water (0.4 cc). Constant duration (4.0 s) heat pulses are presented at quasi-random intervals both during and between trials. Responses by the monkey do not have any effect on the presentation of thermal stimuli. The monkey is randomly exposed to the occurrence of both innocuous (37°-43°C) and noxious (45°-49°C) temperature shifts.

Major Findings: Neuronal activity has been correlated with the following behavioral events: 1) panel light onset indicating the start of a trial; 2) panel press; 3) temperature onset; 4) temperature termination; 5) onset of the light cue in the second task; 6) panel release. Two classes of neurons have been studied. Receptive fields of both classes of neurons were usually limited to the ipsilateral face. Nociceptive neurons exhibited responses correlated with onset and termination of thermal stimuli. In both behavioral tasks thresholds often were below 45°C, and stimulus-response functions were positively accelerating in the noxious heat range. However, the sensitivity of these neurons to thermal stimuli was contingent upon the behavioral situation. Behaviorally non-relevant thermal stimuli presented between trials in the thermal task evoked longer latency responses which were usually reduced in magnitude. Responses to behaviorally non-relevant thermal stimuli presented during the light task were also reduced in magnitude, as compared to stimuli presented during the thermal task. Increasing visual vigilance demands by reducing the intensity of the light cue further reduced the amplitude of heat-evoked neuronal activity. Finally, a warning signal predicting the onset of a noxious (47°-49°C) heat stimulus decreased heat-evoked neuronal activity during the thermal task, as compared to unsignaled trials. Task-related neurons exhibited a maintained discharge throughout the panel press period in both behavioral tasks, although the discharge was usually reduced in magnitude in the light task. Discharge rate was independent of thermal stimulus intensity except for increased responses to 47°C and 49°C noxious heat stimuli in the thermal task. However, this increased response was better correlated with panel release than with stimulus onset. A similar burst of neuronal activity occurred just prior to panel release in warming trials. In the light task, the burst occurred but was somewhat reduced in magnitude when compared with warming trials. The burst was absent if the monkey failed to detect the cue and released the panel inappropriately. The neural discharge occurred following experimenter-initiated trials, when monkey hand movements were not present at panel release. The burst could be temporally dissociated from lip activity in preparation for reinforcement intake by delaying the presentation of the water reward until 2.0 s after a correct panel release. Lip activity was monitored by recording EMG activity from the lip musculature simultaneously with the recording of neural responses. Finally, the burst of neural activity prior to panel release eventually disappeared when reinforcement was withheld during experimenter-initiated trials. Nociceptive neurons exhibited press-related and release-related neurons. In the case of nociceptive neurons, however, this task-related activity was small in magnitude when compared to the stimulus-related activity of these neurons. These data suggest that (1) nociceptive neurons provide information about the intensity of noxious heat stimuli, (2) the activity of nociceptive neurons in nucleus caudalis can be modulated by behavioral contingencies, and (3) the activity of both nociceptive and task-related neurons may be influenced by attentional processes.

Significance to Biomedical Research and Program of the Institute: These studies have successfully identified nociceptive neurons in trigeminal nucleus caudalis--that is, neurons which could transmit information about noxious stimuli and thus possibly participate in the perception of and responses to oral-facial pain. Since these data were collected in awake, behaving animals, our observations extend and modify previous work in acute, anesthetized preparations. Notably, certain classes of nociceptors described for acute preparations respond similarly when tested with stimuli passively administered to awake monkeys. However, when the monkey performs the task, neurons described as potential nociceptors in anesthetized preparations are classified as task-related or nociceptive neurons in awake behaving monkeys. Thus descriptions of the functional properties of potential nociceptive neurons in anesthetized preparations are incomplete. These studies have also shown that the responses of nociceptive neurons can be modified by environmental and behavioral contingencies. These data strongly suggest that the neural representation of oral-facial nociceptive input can be modified at the earliest stage of central nervous system integration. In addition, this work could suggest non-pharmacological approaches to the control of dental pain. Finally, this work has shown promise of providing valuable insights into the roles of such variables as attention, motivation and expectancy in the neural representation of somatosensory stimuli.

Proposed Course: Future experiments include the following: 1) antidromically activating neurons from chronically implanted thalamic stimulating electrodes; 2) shifting the temperature values which distinguish reinforced from non-reinforced trials. Shifting the present $43^{\circ}C$ vs $45^{\circ}C$ comparison to a $45^{\circ}C$ vs $47^{\circ}C$ comparison, for example, could influence stimulus-response functions and thresholds observed for nociceptive neurons. 3) introducing a new behavioral task designed to maximize the sensory-discriminative functioning of monkeys. This task will require monkeys to attend to stimulus intensity in order to chose the greater of two stimuli which are close in magnitude. This new task should maximize the responses of nucleus caudalis neurons to noxious and innocuous thermal stimuli and will allow us with greater reliability to implicate certain classes of nucleus caudalis neurons in pain discrimination.

2. Publications:

Dubner, R. and Hayes, R.L.: Pain mechanisms in the trigeminal system. In: Mechanisms of Pain and Analgesic Compounds, edited by R.F. Beers and E.G. Bassett, Raven Press, New York, 1979, pp. 157-169.

Dubner, R.: Peripheral and central mechanisms of pain. NIH Symposium, in press.

Hayes, R.L., Price, D.D. and Dubner, R.: Behavioral and physiological studies of sensory coding and modulation of trigeminal nociceptive input. In: Advances in Pain Research and Therapy, Vol. 3, edited by J.J. Bonica et al, Raven Press, New York, 1979, pp. 219-243.

Dubner, R. and Price, D.D.: Mechanisms of oral-facial pain. In: Oral Physiology and Occlusion, edited by J.H. Perryman, Pergamon Press, New York, 1978, pp. 193-205.

Dubner, R., Hayes, R.L. and Hoffman, D.S.: Neural and behavioral correlates of pain in the trigeminal system. In: Pain, edited by J.J. Bonica, Raven Press, New York, 1979, pp. 63-72.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 DE 00165-04 NA

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Neuropharmacological Analysis of Supraspinal and Narcotic Modulation of Pain

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Hayes, Ronald L.	Staff Fellow	NIDR NA
	Dubner, Ronald	Chief, NAB	NIDR NA

COOPERATING UNITS (if any)

LAB/BRANCH
Neurobiology and Anesthesiology Branch

SECTION
Neural Mechanisms Section

INSTITUTE AND LOCATION
NIDR, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.45	0.35	0.10

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The major concern of this project was twofold: 1) to develop a primate, behavioral model of pain in which the animal's response to noxious stimuli is primarily, if not totally, dependent upon the sensory (intensity) dimension of pain while independent of the affective dimension of pain; 2) to test the hypothesis that narcotic analgesics (viz, morphine) can suppress the sensory (intensity) dimension of noxious heat stimuli. A corollary of this hypothesis that altering the affective state by minor tranquilizers, like diazepam, is not sufficient to suppress the sensory (intensity) dimension of pain was also tested. Techniques employed in this study were application of thermal heat via a contact thermode, using latencies as a behavioral index of perceptual sensitivity, i.m. injections of drugs, and real-time computer control of the experimental sessions. The objectives of this study were to develop a more comprehensive behavioral model of pain in animals (primates) and to broaden our understanding of the analgesic action of narcotics.

PHS-6040
(Rev. 10-76)

1. Project Description:

Objectives: The general objective of this project was to extend the behavioral model of pain developed earlier by Dubner, Beitel, and Brown (1976) so that the sensory-discriminative (intensity) component and the motivational-affective (aversive) component of the pain experience could be separated from each other. The sensory-discriminative dimension defines such stimulus characteristics as intensity, duration, and location. The motivational-affective dimension delineates emotional qualities, such as anxiety, fear, aversiveness, or unpleasantness, associated with the stimulus.

Methods Employed: The experiments evaluated the relative effects of morphine and diazepam on responses in a task requiring two male rhesus monkeys to attend to the sensory-intensity component of innocuous and noxious thermal stimuli. The animals had to concurrently perform two types of tasks: A detection task (reinforced response) and a discrimination task (nonreinforced response).

Major Findings: We have reported previously that morphine significantly increased the latencies at which monkeys discriminated 47°C and 49°C stimuli from innocuous temperatures of 37-43°C. Diazepam, in contrast to morphine, did not consistently reduce the discriminability of noxious heat (45-49°C) stimuli. In a second experiment, in addition, discrimination of 41° and 43°C stimuli was required. Morphine again suppressed the discrimination of noxious heat stimuli (45° and 49°C), and also increased discrimination latencies to 41° and 43°C stimuli. Diazepam had no systematic effect in this second experiment.

We concluded that the morphine effect was probably related to its attenuation of the perceived stimulus intensity apart from its effect on aversive aspects of the stimulus since the effects were not limited to noxious stimuli.

Significance to Biomedical Research and Program of the Institute: A long standing need in basic pain research has been an animal model of pain that can reflect the different attributes of pain perception. In the past most neurophysiological studies have been done in anesthetized, acute preparations. However, it is evident that recordings from such preparations do not yield an accurate picture of the neural activity of the normal, awake animal. Thus, it has become necessary to record from awake, behaving animals. In awake animals the behavioral model is important because it defines the situation and allows the investigator to relate the neural activity to a specific behavior. The development of animal behavioral models of pain also has importance independent of neurophysiological recording studies. They make it possible to study the psychological mechanisms of pain and its relief. This study, for example, particularly focused upon the mechanism of morphine analgesia.

By showing that morphine can attenuate the sensory dimension it provides a rationale for developing new methods of analgesia which focus upon sensory attenuation rather than influencing the affective dimension of pain.

Proposed Course: This animal model presently is being used in combined behavioral and neurophysiological studies of neural activity in the trigeminal brain stem nucleus. The results of the morphine-diazepam study presently are being prepared for publication.

2. Publications:

Hayes, R.L., Bennett, G.J., Newlon, P.G. and Mayer, D.J.: Behavioral and physiological studies of non-narcotic analgesia in the rat elicited by certain environmental stimuli. Brain Research, 155:69-90, 1978.

Hayes, R.L., Price, D.D., Bennett, G.J., Wilcox, G.L. and Mayer, D.J.: Differential effects of spinal cord lesions on narcotic and non-narcotic suppression of nociceptive reflexes: further evidence for the physiologic multiplicity of pain modulation. Brain Research, 155:91-101, 1978.

Hayes, R.L., Price, D.D., Ruda, M.A. and Dubner, R.: Suppression of nociceptive responses in the primate by electrical stimulation of the brain on morphine administration: behavioral and electrophysiological comparisons. Brain Research, 167:417-421, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 DE 00245-02 NA

PERIOD COVERED
October 1, 1978 to September 30, 1979 CT 0060117

TITLE OF PROJECT (80 characters or less)

Sensations Produced by Tooth Pulp Stimulation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	McGrath, Patricia A.	Biologist	NIDR NA
OTHER:	Gracely, Richard H.	Research Psychologist	NIDR NA
	Heft, Marc W.	Clinical Assoc (Dent)	NIDR NA
	Dubner, Ronald	Chief, NAB	NIDR NA

COOPERATING UNITS (if any)

LAB/BRANCH
Neurobiology and Anesthesiology Branch

SECTION
Neural Mechanisms Section

INSTITUTE AND LOCATION
NIDR, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.80	0.60	0.20

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The objective of this project is to determine the nature of sensations produced by tooth pulp stimulation. Non-pain, as well as pain sensations are evoked when low intensity electric current is applied to human teeth. In order to assess the role of non-pain sensations in the pulp--a traditionally exclusive pain system, these sensations were studied both psychologically and physiologically: 1) the minimum levels of current necessary to produce non-pain and pain sensations were determined for different frequencies of stimulating current; 2) the intensities of sensations from detection threshold to pain threshold were scaled by magnitude production and by verbal descriptors; 3) electromyographic (EMG) activity of the masseter inhibitory period was recorded during tooth pulp stimulation at both non-pain and pain currents; 4) the effect of a narcotic on sensations produced by tooth pulp stimulation and on the masseter inhibitory period was evaluated; and 5) the effects of a conditioning electrical stimulus, applied to the tooth, on sensation and on masseteric inhibitory period were determined.

PHS-6040
(Rev. 10-76)

1. Project Description:

Objectives: The purpose of these studies is to investigate sensations evoked by electrical tooth pulp stimulation. The tooth has been assumed to be an exclusive pain or nociceptive system, and thereby a unique model for the study of pain, pain pathways, and control agents. However, a wide variety of non-pain sensations (such as warmth, tingling, and pressure) is experienced when low intensity electric current is applied to human teeth. The existence of non-pain sensations, in addition to pain sensations, may indicate the presence of a sensory system distinct from a pain system, or these non-pain sensations may simply be a paresthesia and result from near threshold stimulation of an exclusive pain system. If there are 2 distinct sensory systems, there may be consistent differences between the levels of current sufficient to produce sensation and pain-assuming different thresholds for non-pain and pain nerve fibers. Or, the 2 sensory systems may differ in another neural property, temporal summation. Temporal summation can be studied by varying the frequency of stimulation (that is, the number of pulses within a stimulus train) and noting whether there is a uniform or differential effect on non-pain and pain sensations. If all sensations produced by tooth pulp stimulation have similar threshold and summation properties, it is probable that all sensations result from stimulation of one sensory system.

Another means of studying possible differences in the sensory innervation of the tooth pulp is monitoring the masseter inhibitory period, inhibition of masseter activity during substained contraction that occurs after the tooth pulp is stimulated. This inhibition may provide physiological correlates for the non-pain and pain sensations produced by tooth pulp stimulation. Inhibitory periods produced by currents that produce definite non-pain sensations may differ from those caused by currents that produce pain sensation. In order to assess the value of these inhibitory periods as physiological correlates of sensation: 1) the reliability and stability of EM recordings of masseter activity during tooth pulp stimulation at non-pain and pain currents are determined; 2) the correlation between sensation experienced (non-pain or pain) and the inhibitory period is determined; 3) the effects of narcotic (fentanyl) on sensations experienced and on the inhibitory period are evaluated; and 4) the effects of a peripheral electrical conditioning stimulus on sensation and masseteric inhibitory period are determined.

Methods Employed: Non-pain and pain sensations were produced by electrical stimulation (1 sec trains of monopolar, monophasic, cathodal, 1 msec duration constant current pulses) delivered to the labial and the incisal edge of upper central incisors. Frequency of stimulation ranged from 5 to 500 HZ.

Detection and pain thresholds were determined, and the intensities of sensations between these thresholds were scaled by magnitude production and by verbal descriptors.

For EMG monitoring, upper central incisors were stimulated by electrical pulses of 1 msec duration, at currents ranging from detection threshold to supra-pain threshold. Subjects maintained low or high muscle activity by audio-feedback from surface electrodes placed over the masseter muscles. Activity was monitored at the onset of each pulse in a 30 pulses series; recordings were rectified and averaged.

Major Findings: As reported previously in preliminary observations all subjects reported detection thresholds that were significantly lower than pain thresholds at each of the frequencies studied. Sensory thresholds remained constant, regardless of the frequency of the stimulating current while pain thresholds varied monotonically with frequency with a maximum threshold at 5 Hz and a minimum at 100 Hz.

Temporal summation occurred at higher stimulus currents. A strong current that produced a non-pain sensation at a low frequency, 5 Hz, could produce a pain sensation when the frequency of stimulation was increased to 100 Hz. A more descriptive view of temporal summation was shown when the complete range of non-pain sensations was scaled. Temporal summation occurred at a rate proportional to stimulus intensity; lower currents produced less summation than higher currents. There was a multiplicative relationship between stimulus intensity and frequency, so that the strength of sensation increased both as stimulus current increased, and as frequency increased at higher currents.

Three types of inhibitory periods were evoked in the masseter muscle by tooth pulp stimulation: 1) a single inhibitory period, (S): S had a latency of 10-20 msec and a duration of 19-20 msec; 2) a double inhibitory period, (D): the first period had a latency and duration similar to S, followed by a brief burst of muscle activity and a second inhibitory period with a latency of 40-50 msec and a duration of 10-20 msec; 3) a merged inhibitory period, (M), in which the 2 inhibitory periods of a D seemed to merge. M had a latency of 10-15 msec and a duration of 50-80 msec.

Although previous studies report that the occurrence of a S indicates the arousal of a non-pain sensation produced by low stimulus currents, while the occurrence of a D indicates the arousal of a pain sensation produced by high stimulus currents, these studies demonstrate that no particular configuration of inhibitory period was uniquely associated with pain sensation. In 120 observations for 30 subjects, single inhibitory periods occurred rarely (∿10%) always at currents that produced non-pain; D occurred predominantly (∿50%), at low and moderate currents that produced almost equally non-pain and pain sensations, and M occurred in ∿40% of the observations at high stimulus currents that produced almost equally non-pain and pain sensations.

The threshold for the masseteric inhibitory period coincides approximately (+ 2 uA) with an individual's sensory threshold. The type of (S.D.M.) inhibition is related to the strength of the stimulating current, not to a specific sensation. Masseteric inhibition and sensation are two somewhat independent responses to stimulus intensity.

The independence of masseteric inhibition and sensation was demonstrated clearly by the invariance of the masseteric inhibitory period by the narcotic alteration of pain sensation. The administration of fentanyl (1.7 ug/kg IV) increased significantly sensory and pain thresholds, but did not affect the masseteric inhibitory periods. The masseteric inhibitory period evoked by a particular stimulus current was the same before and after fentanyl, despite the changes in sensation produced by the fentanyl.

This dissociation between sensation and inhibition is also shown by the application of an electrical conditioning stimulus to the tooth. The conditioning stimulus consisted of a train of 30 m sec pulses, delivered at 2 sec intervals to the central incisor. The stimulus current was chosen at the highest value that was tolerable to the subject; usually, but not always, this current produced pain. The application of the electrical conditioning stimulus increased sensory and pain thresholds, without any accompanying changes in the masseteric inhibitory period. Sensation is reduced in the adjacent non-conditioned tooth as well as the conditioned tooth indicating the activation of a descending pain suppressing system. Since the pain suppression, produced in part electrical conditioning, is reversed by the administration of an opiate-antagonist, naloxone (10 mg IV) an opiate-like system is in part involved.

Preliminary investigations indicate that the time course for pain suppression on the conditioned central incisor is different from that on the non-conditioned lateral incisor. Quantitative studies of the differential effects of electrical conditioning are now in progress.

Significance to Biomedical Research and Program of the Institute: In recent years the tooth pulp has replaced the cornea (now known to have thermal and pressure innervation) as an exclusive nociceptive system. Consequently much research has focused on producing pain in the tooth by mechanical, chemical, and electrical stimulation in order to investigate pure experimental pain and various methods of controlling it--drugs, hypnosis, electroanalgesia and acupuncture.

The present results show there is also a wide range of non-pain sensations produced when the tooth pulp is stimulated electrically. Subjects are able to scale the intensities of these non-pain sensations, in addition to the pain sensations usually associated with tooth pulp stimulation. Their scales or ratings may be used to assess the effects of various pain control methods and to determine if they act uniformly or differentially on non-pain and pain sensations.

Electromyographic monitoring of masseter inhibitory activity (following chin tap) has been used as a diagnostic tool for assessing temporomandibular joint dysfunction (TMJ). This inhibitory period (following tooth pulp stimulation) has been used also as a nociceptive reflex, a physiological correlate for pain. However, the present results show that the inhibitory period is not correlated with pain sensation. The inhibition is associated with the intensity of the stimulation, not with sensation. The inhibitory period may be used as an index of the current applied to an un-anesthesized tooth (in an anesthesized tooth, there is no sensation and no inhibitory period), but not as a reliable index of pain. This finding, confirmed by the reduction of pain sensation by a narcotic and by an electrical conditioning stimulus without any change in the masseteric inhibitory period, disqualifies the use of the masseteric inhibitory period as a nociceptive reflex.

Proposed Course: Inhibition of pain sensation in a tooth (produced either by a current applied to the tooth itself or to an adjacent tooth) will be further evaluated. The success of some of the electroanalgesia currently used to relieve pain in dentistry is based in part on this inhibition principle. Yet, the specifics of the lateral sensory inhibition process--a generalized phenomenon common to other sensory systems--are not determined for the tooth. Future research will focus on elucidating this inhibition process in the tooth. By concurrent EMG monitoring of masseter reflex activity, it will be possible to assess the extent to which the sensory inhibition is peripheral or central.

2. Publications:

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 DE 00246-02 NA

PERIOD COVERED

October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Masseteric exteroceptive reflex and sensory responses in MPD patients

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	McGrath, Patricia A.	Biologist	NIDR NA
OTHER:	Gracely, Richard H.	Research Psychologist	NIDR NA
	Dubner, Ronald	Chief, NAB	NIDR NA
	Heft, Marc W.	Clinical Assoc (Dent)	NIDR NA

COOPERATING UNITS (if any)

LAB/BRANCH

Neurobiology and Anesthesiology Branch

SECTION

Neural Mechanisms Section

INSTITUTE AND LOCATION

NIDR, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 1.00	PROFESSIONAL: 0.70	OTHER: 0.30

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The objectives of this project are: to establish sensory and pain thresholds to electrical tooth pulp stimulation in myofascial pain dysfunction (MPD) patients and to relate sub-sensory, sensory and pain producing stimuli of the tooth pulp to the masseteric inhibitory period in MPD patients, by means of electromyographic (EMG) recordings. In both these cases comparison between MPD patients to normal subjects will enable a better understanding of the MPD syndrome pain and dysfunction mechanisms, and the gain of insight into possible protective mechanisms of the inhibitory period.

PHS-6040
(Rev. 10-76)

1. Project Description:

Objectives: The purpose of the present study is to measure and correlate the masseteric inhibitory period produced at different biting forces by electric tooth pulp stimulation of non-pain and pain producing intensities in patients with the myofascial pain dysfunction syndrome (MPD).

Proprioceptive (chin-tap) or exteroceptive (e.g., tooth pulp) stimulation produces an inhibition in the electromyographic (EMG) activity of the human masseter during sustained contraction.

MPD patients have a significantly longer inhibitory period after a chin tap than normal controls. This has usually been explained on the basis of muscle spasm and a peripheral mechanism of prolonged discharge from Golgi tendon organs and muscle spindles.

Chin tap in MPD patients also could have produced pain more often than in normal subjects. Thus, pain producing stimuli could have been the cause for the prolonged inhibitory period in MPD patients. The present study will examine the exteroceptive inhibitory period produced by electric tooth pulp stimulation and the correlation between the inhibitory period and different intensity stimuli at subsensation, non-pain and pain producing levels. This will enable us to relate the inhibitory period to pain and non-pain exteroceptive reflexes in the MPD patient as compared to normal subjects. Also, the correlation between inhibitory period and biting forces will be examined to determine the effect of biting force on the latency and duration of the inhibitory period.

Methods Employed: Upper central incisors were stimulated by electric pulses of 1 msec duration at currents ranging from detection threshold (sensory or inhibition) to above pain threshold. Subjects maintained low or high muscular activity by auditory feedback during a train of 30 pulses. Muscle activity was monitored at the onset of each pulse, and EMG recordings were rectified and averaged.

Major Findings: The masseteric inhibitory period evoked by electrical tooth pulp stimulation in MPD patients does not differ from that produced in normal subjects, with respect to latency, duration or configuration. However, MPD patients had thresholds for the masseteric inhibitory periods, that were significantly lower than their sensory thresholds. In normal, healthy subjects, the thresholds for inhibition were within ±2 uA of sensory thresholds. Also, there was more of a tendency towards cyclical or rhythmical grouping action in the masseter muscles of MPD patients. Such cyclical activity has been seen in the masseter muscle of normal subjects, only when they are in an anxious state prior to an oral surgery procedure. These subjects had received either a short-acting narcotic, fentanyl, (1.7 ug/kg IV) or saline, in a double blind paradigm. Their EMG recordings occasionally (25%) showed a tendency towards grouping.

Significance to Biomedical Research and Program of the Institute: The inhibitory period has been found to be significantly longer in MPD patients. This has been attributed to muscle spasm and a peripheral mechanism of prolonged inhibitory discharge from Golgi tendon organs and muscle spindles. A central motorneuron pool mechanism could not, however, be excluded. The present study demonstrates a normal exteroceptive inhibitory period in MPD patients which suggests that the disturbance is not in the central motorneuron pool. The etiological concept associated with a disturbed exteroceptive feedback due to a "pathogenic" occlusion seems unlikely in light of these new findings. This will have further implications in the treatment of the syndrome.

Proposed Course: This study has been completed and a manuscript is being written for publication.

2. Publications:

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 DE 00247-02 NA

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Cytomorphology of functionally characterized spinal cord dorsal horn interneurons

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Bennett, Gary J.	Postdoctoral Fellow	NIDR NA
OTHERS:	Abdelmoumene, Mohammed	Visiting Scientist	NIDR NA
	Hoffert, Marvin J.	Senior Staff Fellow	NIDR NA
	Dubner, Ronald	Chief, NAB	NIDR NA

COOPERATING UNITS (if any)

LAB/BRANCH
Neurobiology and Anesthesiology Branch

SECTION
Neural Mechanisms Section

INSTITUTE AND LOCATION
NIDR, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 2.70	PROFESSIONAL: 2.35	OTHER: 0.35

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Neurons in the spinal cord dorsal horn of monkeys and cats, especially neurons in lamina I and the substantia gelatinosa (SG), have been functionally characterized and intracellularly stained with horseradish peroxidase. The dendritic arbors and axonal extensions of these functionally identified neurons have been examined at the light microscope level. Wide dynamic range neurons, responsive to innocuous stimuli but activated maximally by noxious stimuli are found in layers I-V of the dorsal horn and have more extensive arbors than neurons in the same layers activated only by noxious stimuli (nociceptive-specific). The cytomorphological analysis of these functionally characterized neurons will soon be extended to the level of resolution of the electron microscope.

PHS-6040
(Rev. 10-76)

1. Project Description:

Objectives: The small interneurons of the substantia gelatinosa (SG, Rexed's lamina II) are believed to be involved in the integration and modulation of somatosensory primary afferent input. These cells may be particularly involved in pain sensation. Extracellular electrophysiological analyses of SG neurons have recently appeared. The extracellular method, however, does not allow one to unambiguously identify SG neurons, since this area also contains the apical dendrites of neurons whose perikarya lie beneath the SG. Moreover, the extracellular method can not identify which of the several morphological types of SG neurons the physiological recordings emanate from.

We have physiologically characterized individual SG neurons and then intracellularly injected the same neurons with the enzyme, horseradish peroxidase (HRP). This enzyme spreads throughout the cell's interior and, when exposed to certain histochemical reagents turns black; thus visualizing the cell and its processes. This technique obviously allows the neuron in question to be precisely located. Importantly, the technique also reveals the neurons morphology. Because the HRP reaction product is electron opaque, the morphological characterization can be extended to the ultrastructural level. Using this methodology, we have begun a detailed examination of the physiology and morphology of the somatosensory neural circuitry in the SG.

Methods Employed: The lumbosacral spinal cords of anesthetized adult cats are prepared for electrophysiological recording in the usual manner. We use glass micropipettes with tip diameters of about 1.0 um. Because these relatively large tips yield poor intracellular records, we physiologically characterize the neuron before penetration. The cell's responses to a battery of natural stimuli (touch, hair, movement, pressure, pinch, noxious heat, etc), and to percutaneous electrical stimulation of the cell's receptive field are determined. The percutaneous electrical stimulus allows us to estimate the conduction velocities of the cell's inputs. After physiologically characterizing the neuron, an attempt is made to introduce the micropipette into the neuron's interior. Successful penetrations are carefully checked in order to be certain that the impaled neuron is the same as the one characterized. HRP is iontophoresed into the cell for the duration of the impalement (1-15 min). At the conclusion of the experiment, the animal is perfused with fixative and the spinal cord removed. The cord is sectioned parasagittally into 150 um thick sections. The tissue is then incubated in 0.5% $COCL_2$ and the HRP reaction product is developed by incubating with diaminobenzidine and hydrogen peroxide. The developed sections are cleared with glycerin and examined with the light microscope. Camera lucida drawings (1,650 X) are prepared for well stained examples. The tissue is then removed from the glycerin and processed for electron microscopy in the usual fashion.

Major Findings: Our results have confirmed that there are two common morphological types of gelatinosa neurons. These neurons, the stalked cell and the islet cell, have been previously described in the medullary gelatinosa. The stalked cells have their perikarya situated along the dorsal border of the SG. Their dendrites travel ventrally for variable distances with oblique angles of descent while simultaneously spreading out in the rostrocaudal plane. Their dendritic arbors are thus fan-shaped. Although the arbors are extensive rostro-caudally and to a variable extent dorsoventrally, they are compressed mediolaterally. The stalked cell's axons arborize extensively in layer I. We have found that stalked cells physiological responses are of two general types. Nociceptive specific (NS) neurons respond only to noxious stimuli. Wide dynamic range neurons respond to both innocuous mechanical stimuli (e.g., hair movement) and to noxious stimuli. Ultrastructural analysis of a HRP filled stalked cell has shown that this cell type receives a heavy innervation from primary afferents and that its axon synapses in the vicinity of layer I dendrites. It has been hypothesized that stalked cells are excitatory interneurons that relay primary afferent input to layer I neurons. Our observation that the physiological responses of NS and WDR stalked cells are very similar to the physiological responses of layer I neurons (which also occur as either NS or WDR neurons) supports this hypothesis.

Islet cells have perikarya that lie in the midst of their dendritic arbors. The dendrites travel predominantly in the rostrocaudal axis within a narrow dorsoventral band. These arbors are also compressed mediolaterally. The axons generally create a dense plexus within the territory of the dendritic tree, but may extend well beyond the rostrocaudal limits of the dendritic arbor while maintaining the same relative dorsoventral position. Our data indicate that there are two types of islet cells. Layer IIa islet cells have dendritic arbors with rostrocaudal expanses of 410-880 um. Nearly all of the dendrites are contained within layer IIa. Physiologically, these cells are either NS or WDR neurons. The islet cells whose dendrites are mostly contained within layer IIb have rostrocaudal expanses of 1,200-1,400 um. Their physiological responses indicate that they are innervated only by low threshold mechanoreceptive (LTM) primary afferents. Electron microscopic observations of HRP filled layer IIa islet cells show that the dendrites of these cells contain aggregates of synaptic vesicles. These vesicle filled dendrites participate in several types of synapses, e.g., they are presynaptic in dendrodendritic synapses onto dendrites that do not contain vesicles (these may be stalked cell dendrites). They are also presynaptic in dendroaxonic synapses onto the primary afferent endings from which they receive some of their inputs. The islet cells are believed to be inhibitory interneurons. The ultrastructural observations are certainly congruent with this hypothesis. Our finding that there are differences between layer IIa and layer IIb islet cells suggests that each type may be involved in separate inhibitory phenomena.

Significance to Biomedical Research and the Program of the Institute: The neuronal circuitry that subserves the sensation of pain is only dimly elucidated. The design of rational therapies for the amelioration of neuropathological syndromes that present disturbed pain sensation as their most salient symptom requires an accurate understanding of the neural mechanisms that mediate normal pain sensation. This project aims toward an unambiguous and detailed elucidation of the physiology and morphology of the neuronal circuitry of the SG.

Proposed Course: We intend to continue to examine physiologically characterized neurons with both the light and electron microscopes. In addition to neurons in the SG, we will extend our analysis to the neurons immediately beneath the SG in the nucleus proprius. Moreover, we hope to be able to employ immunohistochemical techniques in order to identify the neurochemistry of the HRP filled neurons and their connections.

2. Publications:

Bennett, G.J., Hayashi, H., Abdelmoumene, M. and Dubner, R.: Physiological properties of stalked cells of the substantia gelatinosa intracellularly stained with horseradish peroxidase. Brain Research, 164:285-289, 1979.

Price, D., Hayashi, H., Dubner, R. and Ruda, M.A.: Functional relationships between neurons of the marginal and substantia gelatinosa layers of the primate dorsal horn. J. Neurophysiol., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 DE 00020-14 NA

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Anatomical studies of the main sensory and spinal trigeminal nuclei

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Gobel, Stephen	Chief, NEA Section	NIDR NA
Coinvestigators:	Falls, William	Senior Staff Fellow	NIDR NA
	Arvidsson, Jan	Visiting Fellow	NIDR NA
	Brown, Emma	Bio. Lab. Tech. (Ele. Mic)	NIDR NA
	Allen, Barbara M.	Biologist	NIDR NA

COOPERATING UNITS (if any)

LAB/BRANCH
Neurobiology and Anesthesiology Branch

SECTION
Neurocytology and Experimental Anatomy Section

INSTITUTE AND LOCATION
NIDR, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.10	1.85	1.25

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project is concerned with the synaptic connections of the sensory root of the trigeminal nerve and the morphology of the neurons which comprise the main sensory and spinal trigeminal nuclei. These studies employ electron microscopy, the Golgi method and the use of intraneuronal markers such as horseradish peroxidase. The objects of these studies are to delineate trigeminal pain-temperature pathways and to broaden our understanding of oral-facial sensation.

PHS-6040
(Rev. 10-76)

1. Project Description:

Objectives: The objectives of our anatomical studies during the past year were twofold. First, to fill small caliber primary axons which respond to noxious stimuli with the protein marker horseradish peroxidase (HRP). This marker can be visualized both at the light and electron mocroscopical levels and provides an opportunity to determine the laminar distribution of these axons within the substantia gelatinosa (SG) and characterize their terminal axonal arborizations. Second, to examine the synaptic connections of the two major interneurons of the SG which have been impaled with a micropipette had their primary inputs characterized and subsequently filled with HRP.

Methods Employed: Detailed camera lucida drawings were made of HRP-filled neurons at 1,000 X using the light microscope which depicted their axonal and dendritic morphology, branching pattern and laminar distribution. After completion of the drawings, the sections containing the HRP-filled neurons were processed for electron microscopy in order to study their fine structural characteristics and their synaptic connections.

Major Findings: The SG, which caps the dorsal horn in the medulla and spinal cord, is a major termination site of primary trigeminal and spinal neurons respectively which respond to noxious stimuli. Individual small caliber (1.0-1.5 μm) myelinated primary axons enter layers IIa and IIb where they give rise to as many as five fine (0.1-0.2 μm) axonal strands which are extended for several hundred μm in the rostrocaudal axis of these layers. Each of these strands bear numerous bead-like axonal endings which lie in the center of the layer IIa and IIb glomeruli where they synapse on two different kinds of dendrites, i.e., those which contain synaptic vesicles and are sources of as well as receivers of synaptic inputs (type 2 dendrites), and those without synaptic vesicles which only receive synaptic input (type 1 dendrites). These findings confirm part of the hypothesis which arose from our tooth pulp extirpation studies which stated that layer II is a major termination site of primary neurons with small myelinated axons while layer I is a major termination site of primary neurons with unmyelinated axons.

The axonal endings of the primary axonal strands in layer II synapse on the dendrites of two major kinds of interneurons, i.e., neurons whose axons arborize within SG and do not project to higher levels of the neuraxis. In earlier EM analyses, we have been able to thoroughly characterize the kinds of dendrites which receive synaptic input from primary endings in layer II. However, it had not been possible to determine which kinds of dendrites belong to which of the many cell types that make up the neuropil of SG largely because all of the dendrites are about the same size and shape. During this past year, our colleagues in the Neural Mechanisms Section have succeeded in iontophoretically injecting the two major SG interneurons, i.e., stalked cells and islet cells with HRP. Having the dendrites and axons of these neurons marked with HRP has provided an opportunity to study their synaptic connections in detail.

The stalked cell has its cell body along the I/IIa border and gives rise to a cone-shaped dendritic arbor which traverses layers IIa and IIb while its axon enters layer I. The layer IIa islet cell gives rise to extensive rostral and caudal dendritic arbors which extend for several hundred μm in layer IIa. Its highly branched axonal arbor generates numerous branches within the confines of the islet cell dendritic arbors in typical Golgi type II fashion.

The EM analysis of these cells revealed that both cell types received asymmetrical axodendritic synapses from primary endings in the layer IIa glomeruli and widely separated symmetrical synapses from small non-primary endings outside of the glomeruli. The former inputs activate or bring these cells closer to firing action potentials while the later inputs are thought to arise from the serotonergic neurons in the brain stem and inhibit or decrease the output of these cells.

The major difference between these two cell types is that the islet cell contains clusters of synaptic vesicles in their spine heads (type 2 spines) and dendritic shafts while stalked cells do not. Islet cell dendrites form symmetrical synapses on several different kinds of neural processes. Within the glomeruli, they form dendrodendritic synapses on type 1 spines and on other small dendritic shafts and dendroaxonic synapses on primary endings. Outside of the glomeruli, islet cell dendrites also form dendrodendritic synapses on type 1 spines and different sized dendritic shafts and dendroaxonic synapses on the shafts of unmyelinated axons. The axonal endings of the unmyeliated axon of the islet cell forms symmetrical synapses on layer II dendritic shafts and spines.

In summary, light and EM analyses of primary axons and the two major interneurons of layer IIa which have been marked with HRP so that they stand out from the other neural consistuents have provided several important advances in our understanding of the neural events which take place at the first synapse in trigeminal and spinal pain pathways. The primary axons and the two interneurons in layer IIa are thought to function in the following manner. Axonal endings of primary neurons with small myelinated axons activate stalked cells, excitatory interneurons whose axons synapse on the dendrites of second order projection in layer I. The layer IIa islet cell, functions as an inhibitory interneuron, i.e., it inhibits or diminishes the transfer of primary inputs to the layer I projection neurons via the stalked cell through (1) its dendrodendritic synapses on stalked cell dendrites (2) its dendroaxonic synapses on primary endings and (3) its axodendritic synapses on stalked cell dendrites.

Significance to Biomedical Research and the Program of the Institute: The rationale for utilizing orofacial pain in diagnosing dental pathology, for providing anesthesia for dental procedures and for understanding the role of pain in reflex movement of the musculature of the head and neck is based on our knowledge of orofacial pain pathways. Our knowledge of much of these neural pathways today is fragmentary. Our neuroanatomical studies are aimed at establishing a more definitive circuit diagram of trigeminal pain pathways.

The trigeminal nerve is involved in a host of chronic pain states which include trigeminal neuralgia (tic douloureux), glossodynia (burning tongue) and other facial neuralgias. Explanations of these pain states usually involve mechanisms related to pathology of the peripheral nerve. However many of the symptoms of tic douloureux, for example, cannot be explained without considering synaptic circuitry in the central nervous system. For example, why are only the maxillary and mandibular divisions involved i.e., only those divisions supplying the teeth? Why do the most innocuous stimuli trigger the pain episode? Is the loss of teeth somehow involved in a disruption of synaptic connections in the spinal trigeminal nucleus?

Recent technical advances have made it possible to selectively study individual neurons in trigeminal pain pathways at the light and electron microscopical levels. Data from such studies will provide more detailed information about the neural circuitry about trigeminal pain pathways and will permit us to design more critical experiments to approach the above as well as other questions concerning chronic pain states in the orofacial region.

Proposed Course: During the coming year we will use our ability to selectively mark the terminal axonal arbors of primary neurons with HRP in order to critically examine their synaptic connections in the three layers of the SG. This approach is superior to cutting the trigeminal root and looking for degenerating endings because different kinds of primary neurons degenerated at different rates and require many experiments. In addition it will provide an opportunity to examine the intact primary ending rather than degenerating, shriveled, fragmenting endings as seen with degeneration techniques.

We will also reexamine the terminal distribution of primary neurons which innervate tooth pulps using several new approaches. HRP and ferritin will be conjugated to a variety of different plant lectins such as wheat germ agglutinin in attempts to specifically mark the axonal endings of these neurons. Since lectins may be transported orthogradely in primary neurons in greater amounts than HRP it may be possible to obtain a much more accurate picture of the distribution of these neurons than ever before possible. In addition we will continue our study of the transsynaptic degenerative changes in SG neurons following pulpotomies.

Finally, we will continue our analyses of the synaptic connections of the neurons of SG. The EM analyses of the synaptic connections of these neurons should add greatly to our understanding of how primary inputs are processed in the SG.

2. Publications:

Gobel, S.: Neural circuitry in the substantia gelatinosa of Rolando: Anatomical Insights. In: *Advances in Pain Research and Therapy*, Vol. 3, edited by J.J. Bonica. Raven Press, 1979, in press.

Gobel, S. and W.M. Falls: Anatomical observations of horseradish peroxidase filled terminal primary axonal arborizations in layer II of the substantia gelatinosa of Rolando. *Brain Res.*, 1979, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 DE 00166-04 NA

PERIOD COVERED

October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Anatomical Studies of Postnatal Maturation in the Spinal Trigeminal Nucleus

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Falls, William M.	Senior Staff Fellow	NIDR NA
OTHER:	Gobel, Stephen	Chief, NEA Section	NIDR NA

COOPERATING UNITS (if any)

LAB/BRANCH

Neurobiology and Anesthesiology Branch

SECTION

Neurocytology and Experimental Anatomy Section

INSTITUTE AND LOCATION

NIDR, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 0.95	PROFESSIONAL: 0.40	OTHER: 0.55

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

These studies examine the postnatal development of neurons in trigeminal pain pathways in the dorsal horn of the medulla (trigeminal nucleus caudalis) with Golgi and electron microscopic techniques. They compare in newborn kittens the maturity of the two major interneurons in the substantia gelatinosa (the stalked cell and the islet cell) with their adult counterparts. They follow the developmental sequences in the formation of the dendritic and axonal arbors of both stalked cells and the islet cells and examine how these interneurons eliminate excess dendrites as well as how they provide additional plasma membrane for their elongating dendrites and axons. These studies also determine which components of the synaptic circuitry in trigeminal pain pathways are present at birth and which develop postnatally.

1. Project Description:

This project was completed during the fiscal year ending September 30, 1978. During the past year a manuscript was completed and submitted for publication.

A brief summary of the findings of this study follows. The two major interneurons of the substantia gelatinosa of Rolando form their dendritic arbors similarly. Prior to birth, they form numerous short dendrites. A few of these dendrites continue to lengthen while many bead up and are lost. EM analyses of the dendrites of these cells at birth revealed numerous clusters of vesicles (addition vesicles) which become incorporated into the dendritic plasma membrane by fusing with the existing plasma membrane in exocytotic fashion. These fusions are though to represent the way in which elongating dendrites lengthen their plasma membranes. The EM analyses also revealed that many of the dendrites are lost through a process of cavitation in which addition vesicles fuse with the agranular reticulum. The agranular reticulum then enlarges to form large cavities within the dendrites. The membranes and the cavities open to the intercellular space. The dendrites then fragment and disappear. Although the cavitating dendrites receive synapses, they have never been found receiving synapses from primary endings. The failure of such synapses to materialize by a certain critical time in the dendrite's development is thought to result in the beginning of the cavitation process and in the ultimate destruction of the dendrite.

This hypothesis led to important studies in adult animals in which the receptors of primary neurons innervating tooth pulps were extirpated and prevented from regenerating. The removal of inputs from these primary neurons led to the initiation of cavitation in the dendrites of adult stalked cells and islet cells. Small cavities formed and subsquently enlarged, hollowed out the dendrites, fused with the dendritic plasma membrane similar to the cavitation sequence in the developmental situation. These experiments indicate that primary inputs to these cell types are essential not only to the development of their dendritic arbors but also to their maintenance throughout adulthood. They also suggest that disease related or traumatic injuries to peripheral nerves in which the peripheral nerves do not reestablish their receptors can be expected to result in severe transsynaptic changes in neurons in the central nervous system subserving pain pathways. Such disruptions in the normal neural circuitry of pain pathways may result in the formation of chronic pain states.

2. Publications:

Falls, William M. and Gobel, Stephen: Golgi and EM Studies of the Formation of Dendritic and Axonal Arbors: The Interneurons in Layers IIa and IIb of the Dorsal Horn of the Medulla. J. Comp. Neur., 187: 1-18, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 DE 00207-03 NA

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Pharmacological Characterization of Axonal Endings in Trigeminal Nucleus Caudalis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Ruda, Maryann T.	Staff Fellow	NIDR NA
OTHER:	Gobel, Stephen	Chief, NEA Section	NIDR NA

COOPERATING UNITS (if any)

LAB/BRANCH
Neurobiology and Anesthesiology Branch

SECTION
Neurocytology and Experimental Anatomy Section

INSTITUTE AND LOCATION
NIDR, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.65	1.15	0.50

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This study attempts to pharmacologically characterize axonal endings in the dorsal horn of the medulla (DHM). The methods employed include light and electron microscopic autoradiographic localization of labeled axonal endings in the DHM following application of tritiated $[^3H]$ neurotransmitters onto the DHM and immunohistochemical localization of specific populations of axonal endings. The neurotransmitters being investigated include serotonin, norepinephrine, enkephalin and substance P. The purpose of these studies is to locate and morphologically characterize pharmacologically distinct axonal endings in the DHM which might be involved in trigeminal pain pathways.

1. Project Description:

Objectives: The purpose of these experiments is twofold: 1) to characterize the ultrastructural morphology of pharmacologically identified axonal endings in the substantia gelatinosa (SG) of the dorsal horn of the medulla (DHM); 2) to develop a pharmacologically characterized circuit diagram of potential synaptic interactions in the DHM. Our initial studies focused on the monoamines serotonin (5HT) and norepinephrine (NE). The data on 5HT terminals are in press while those on NE terminals are presently being analyzed and prepared for publication. Having morphologically identified the monoaminergic terminals in SG, we are presently investigating raphe magnus and locus coeruleus afferents to SG in an attempt to correlate the morphology and synaptic interactions of the monoaminergic terminals we have identified, with their nuclear origin. This type of correlation is important in that it describes potential neural circuits through which stimulation produced analgesia can act. Other experiments which are in their initial stages will examine different peptidergic axonal endings in SG.

Methods Employed: Pharmacological characterization of cell bodies and axonal endings in our studies involve three different techniques. The first technique utilized in studying monoamines involved light and electron microscopic autoradiography. The approach relies on the ability of axonal endings which normally utilize a specific neurotransmitter, to take up this same neurotransmitter when it is exogenously applied in a radioactive form. In our experiments, either [^{3}H] 5HT or [^{3}H] NE was topically applied onto the DHM. Following fixation to bind the monoamines, the DHM was processed for autoradiography. Slides carrying 1 u or EM sections were coated with liquid nuclear emulsion, exposed in the dark for several weeks to months and subsequently treated with a photographic developer. This process results in the deposition of silver grains over areas containing the [^{3}H] neurotransmitter and thus localize specific axonal endings. In other experiments an [^{3}H] amino acid was injected into nucleus raphe magnus and the locus coeruleus and anterograde transport of the radioactive label to SG was used to mark the axonal endings.

A second technique utilized in our experiments involved the serotonin neurotoxin, 5,6 dihydroxytryptamine (5,6 DHT). This neurotoxin destroys 5HT terminals, resulting in morphological changes indicative of degeneration. These changes can be seen at the EM level. Experiments in which both 5,6 DHT and [^{3}H] NE are used, provide two distinguishable EM markers so that interactions of both monoamines can be compared in the same experiment.

A third method employs antibodies raised to a specific neurotransmitter to immunohistochemically localize peptide neurotransmitters at light and EM levels.

Major Findings: Light microscopic analysis of the distribution of silver grains in 1 μ sections of the DHM demonstrates the presence of both [^{3}H] 5HT and [^{3}H] NE in layers I and II. Silver grains occur both as individual grains and as aggregates composed of many individual grains. In comparing the density of silver grains in SG in the [^{3}H] 5HT and [^{3}H] NE experiments, there is a greater amount of both individual grains and grain aggregates following application of [^{3}H] 5HT. Neither [^{3}H] 5HT nor [^{3}H] NE labeled neuronal cell bodies have been found in the DHM.

Electron microscopic analysis of axonal endings labeled with [^{3}H] 5HT demonstrates two categories of morphologically distinguishable endings: dome-shaped endings which form a single synapse and scalloped endings which form multiple synapses. Each category can be further divided into several types based on morphological criteria. The [^{3}H] 5HT labeled endings synapse primarily on small caliber dendritic shafts and spines, with the dome-shaped endings forming both symmetrical and asymmetrical synapses and the scalloped endings forming only symmetrical synapses. Dome-shaped endings were most common and two types were found in layer I and II while a third type was found only in layer II. Layer I contained a single type of scalloped ending while layer II contained three types of scalloped endings.

The presence of serotonergic endings in layer I suggests that some of these endings synapse on the dendrites of layer I projection neurons where they may inhibit the output of the projection neuron directly. Serotonergic endings in layer II may modulate the activity of layer II interneurons by synapsing directly on these interneurons. The interneurons in layer II may function by mediating the transfer of inputs from primary endings in these layers to layer I projection neurons.

Dome-shaped and scalloped shaped [^{3}H] NE labeled endings are also present in the substantia gelatinosa. These categories of endings can be further divided into five types based on morphological criteria. Three types of dome-shaped endings are found in layers I and II and are distinguished by the presence of either pleomorphic, small oval or large oval agranular vesicles. They form mainly asymmetrical synapses on dendritic spines and shafts although some symmetrical synapses and occasional axosomatic synapses are found. In the experiment combining the serotonin-neurotoxin 5,6 DHT and [^{3}H] and [^{3}H] NE uptake, several examples of the same dendrite receiving synapses from both a degenerating 5HT ending and an [^{3}H] NE labeled ending were found. One type of scalloped ending is found in both layers I and II while a second type is found only in the deeper part of layer II. The scalloped endings form asymmetrical synapses on dendritic shafts and spines.

The presence of [^{3}H] NE labeled axonal endings in layers I and II of the DHM suggests that NE afferents have access to both the projection neurons in layer I and the interneurons in layer II. The observation of both 5HT and NE endings synapsing on the same dendrite further suggests that both monoaminergic systems play similar roles in the modulation of the output of layer I projection neurons.

Significance to Biomedical Research and Program of the Institute: These experiments provide a circuit diagram of the monoaminergic input to layers I and II of the DHM. The finding of 5HT and NE axonal endings in the DHM is of particular significance since the activation of descending serotonergic and noradrenergic pathways from nucleus raphe magnus and the locus coeruleus are implicated in the production of analgesia. Our experiments demonstrate that the modulation of layer I nociceptive neurons can occur either through direct axodendritic serotonergic and noradrenergic synapses on layer I projection neurons, or by input onto layer II interneurons. With an understanding of the mechanism of action of analgesia produced by the monoamines, the potential exists to activate the system pharmacologically in order to alleviate pain. The pharmacological circuit diagram is also applicable to the spinal cord dorsal horn since the organization of both have been shown to be similar.

Proposed Course: Our analysis of neurotransmitters in layers I and II of the DHM has demonstrated 5HT and NE axonal endings, described their morphology and developed a circuit diagram of potential interactions between descending monoaminergic inputs and intrinsic neurons in the DHM. Experiments utilizing the neurotoxin 5,6 DHT corroborated the presence of 5HT endings and demonstrated that both 5HT and NE act, in part, on the same neuron. Presently, we are extending our analysis of monoamines in the DHM by correlating the nuclear origin of the monoamines axonal endings with the morphological types described in the neurotransmitter uptake experiments. This correlation is important since serotonergic n. raphe magnus and noradrenergic locus coeruleus neurons have been implicated in the production of analgesia. Information on their role in the synaptic circuitry of the DHM would help elucidate the mechanism of action of stimulation produced analgesia.

Future experiments will examine the role of substance P, somatostatin and enkephalin in the neural circuitry of the DHM. In these experiments the technique of immunohistochemistry will be used to identify the neurotransmitter.

The ultimate goal of our series of experiments will be to combine more than one EM marker in the same experiment so that interaction between pharmacologically identified axonal endings and neurons can be demonstrated. Additionally, experiments involving pharmacological analysis of inputs onto physiologically identified and intracellularly stained neurons are also planned. The data from these experiments will lead to a clearer understanding of the pharmacological interactions in the DHM.

2. Publications:

Ruda, M.A. and Gobel, Stephen: Ultrastructural Characterization of Axonal Endings in the Substantia Gelatinosa which take up [^{3}H] Serotonin. Brain Research, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 DE 00248-02 NA

PERIOD COVERED

October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Horseradish peroxidase studies of trigeminal pain pathways

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Hockfield, Susan J.	Biologist	NIDR NA
OTHERS:	Gobel, Stephen	Chief, NEA Section	NIDR NA

COOPERATING UNITS (if any)

LAB/BRANCH

Neurobiology and Anesthesiology Branch

SECTION

Neurocytology and Experimental Anatomy Section

INSTITUTE AND LOCATION

NIDR, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 0.40	PROFESSIONAL: 0.40	OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project examines the neural circuits involved in the appreciation of oral-facial sensations, particularly the circuits mediating pain and temperature sensations. Incoming sensory information may be modified by neuronal activity in many areas of the nervous system before being relayed to higher brain centers. Neuron marking techniques, in which a foreign protein (horseradish peroxidase) is picked up by axonal endings and carried back to their neuronal cell bodies, accurately demonstrate which neurons participate in a given neural circuit. Previous experiments in this study identified the neurons that relay incoming oral-facial sensations to higher brain centers. Experiments conducted during the past year have identified the neurons that can modify incoming sensory information and that may, in part, mediate analgesic and hyperalgesic states.

PHS-6040
(Rev. 10-76)

1. Project Description:

Objectives: The objective of this study is to identify the neurons in the central nervous system that project to the dorsal horn of the caudal medulla and may, in part, modify incoming sensory information from the face. Three general locations of these neurons will be analyzed: (1) within the main sensory and spinal trigeminal nuclei; (2) in areas of the brainstem outside of these trigeminal nuclei; and (3) in the cervical spinal cord.

Methods Employed: Horseradish peroxidase (HRP), a protein of about 40,000 molecular weight was microinjected into nucleus caudalis. This protein is picked up pinocytotically by axonal endings and transported retrogradely through the axon to the cell body of origin where it is sequestered in lysosomes. After the appropriate histochemical reaction, cells of origin which send their axons into nucleus caudalis can be clearly visualized in the brainstem.

Major Findings: The initial experiments of this project identified three locations of trigeminothalamic projection neurons in and adjacent to nucleus caudalis. These are: layer I, layer V, and an area just ventral to nucleus caudalis which represents the trigeminal component of the lateral cervical nucleus. The similarities between the morphology of nucleus caudalis and the spinal cord dorsal horn, and between the locations of trigeminothalamic neurons in nucleus caudalis and of spinothalamic neurons in the spinal cord dorsal horn, has led us to regard nucleus caudalis as the medullary continuation of the head of the dorsal horn of the spinal cord. It receives trigeminal sensory input in the same way that the dorsal horn of the spinal cord receives sensory input for the rest of the body.

In the next series of experiments, by injecting horseradish peroxidase into the dorsal horn of the caudal medulla, we have identified the sources of input to the medullary dorsal horn which are involved in modifying the output from this nucleus to higher brain centers. Neurons in many locations in the spinal cord and brainstem send axons to the medullary dorsal horn. In the spinal cord, neurons that project to the caudal medulla are found predominantly in layers I, III, IV, V and VII. Contrary to classical descriptions, they are not found in layer II. In the caudal medulla, neurons in layers I, IV, V and VII project to the opposite medullary dorsal horn. Neurons in the more rostral trigeminal sensory nuclei and the areas bordering them also send projections into the dorsal horn of the caudal medulla. The main sensory nucleus and the subnuclei of the spinal trigeminal nucleus can be characterized by the density and morphology of their neurons that project to the caudal medulla.

Descending projections to the caudal medulla also arise from neurons in the raphe nuclei, the nucleus locus coeruleus, the reticular formation and the periaqueductal grey matter. Some of these are likely to be the sources of serotonergic and noradrenergic axonal endings in layers I and II that can inhibit trigeminothalamic neurons in the caudal medulla and can produce analgesia.

Significance to Biomedical Research and Program of the Institute: Oral-facial pain is manifested frequently throughout the population in both acute and chronic states. Elucidation of the neural circuits mediating oral-facial pain can demonstrate how acute and chronic pain states occur and may suggest how they can be controlled. Recent evidence indicates that the mechanisms relating to analgesia such as drug therapy, acupuncture, and electrical stimulation of the skin, the dorsal columns, and the periaqueductal gray, may involve some of the circuits demonstrated in these studies. This project has shown some of the neural pathways that are involved in the perception of pain and analgesia.

Proposed Course: The project has now been completed. A manuscript of this work is currently being prepared.

2. Publications:

None

Report of the Clinical Investigations Branch
National Institute of Dental Research
Summary Statement FY 1979

The Clinical Investigations Branch is concerned with the study of factors influencing diagnosis of oral disorders from theoretical to descriptive analyses of known functional relationships.

The Branch is comprised of two sections: the Oral and Pharyngeal Development Section (OPD) under Dr. James Bosma, and the Diagnostic Methodology Section (DMS) headed by Dr. Richard L. Webber. These sections work largely independently. OPD emphasizes the study of form, development and function of oral and pharyngeal tissues through detailed anatomical description and physiological analyses coupled with psycho-physical measurements. DMS approaches the study of oral disorders with more of a systems orientation which is grounded in image processing and information theory. Together the sections complement each other by providing a multidisciplinary approach to diagnostic problems of mutual interest.

Recent activities in OPD have involved the study of sensory, motor and salivary secretory mechanisms. Sensory studies make use of psycho-physical testing procedures to determine detection and recognition thresholds and to quantify suprathreshold perception of sensory stimuli. Of particular interest are processes underlying the perception of salt and sweet taste among normals and individuals having sensory impairment.

Studies of motor function of the oral-pharyngeal complex have focused upon the pharynx and larynx as related to swallow, speech, and airway maintenance. Radiologic data provide the substance for a book, Radiography of the Pharynx and Larynx, which is to be published by Springer-Verlag.

Studies of normal and disease-related salivary functions are also under way to determine the relationship between specific salivary constituents (proteins, specific enzymes) and functional deficit. Diseases of interest include Sjogren's syndrome, aphthous stomatitis, and pancreatic disorders.

Two long-range anatomical projects are nearing completion which provide the substance for two reference books. One, Postnatal Development of the Rat Skull, by M. J. Baer, J. M. Ackerman and J.F. Bosma has been critically reviewed and is being considered for publication by the National Library of Medicine. The other, Anatomy of the Infant Head, by J. F. Bosma has progressed to a point where its illustrations are essentially finished.

DMS activities are balanced between the investigation of new bases for quantitatively classifying information of diagnostic interest and the study of alternative technology via computer simulation and development of prototype systems.

Recent work has centered on application of the symmetric-axis geometry pioneered by Blum to the description of growth and development of the human mandible. This mathematical tool has been used to describe mandibular shape in a way which unequivocally demonstrates a series of invariant angular relationships. These invariants appear to be independent of the age of the individual studied and may be species specific. They also are independent of the coordinate space used to describe the shape.

Other studies deal explicitly with problems associated with the demonstration of impaired performance of explicitly defined diagnostic tasks from various different x-ray systems including a second-generation CAT machine and various prototype dental x-ray systems. In addition to methodological considerations these studies provide rational bases for tailoring the information-carrying capacity of the system to the amount of information required for reliable performance of specific diagnostic tasks.

Hardware development is done largely in collaboration with other Federal agencies. This includes technological evaluation and the development of prototype systems. The application of broad-spectrum rod-anode x-ray sources coupled to various high-speed image-detectors is of particular interest at this time, since isotopic intra-oral sources have been shown to be less than ideal for many fluoroscopic applications. The use of a feed-back system to determine appropriate projection geometry is also being considered in this context in order to facilitate the detection of lesion-changes from one examination to the next.

This has been a year of significant change within DMS with regard to resources and personnel. The entire laboratory facility was relocated into larger quarters and key programming personnel including one senior investigator, Dr. Roger Nagel, transferred to a different program at the National Bureau of Standards. This move parallels an evolutionary trend within DMS, toward more specific scientific applications research now that the basic technological capabilities of the laboratory have been established. Dr. Nagel's replacement as a Senior Staff Fellow is Dr. Waldemar de Rijk, a physicist/dentist with explicit research interest in diagnostic systems.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 DE 00065-08 CI

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Development Evaluation of Improved Dental Radiographic Systems with Emphasis on Factors Influencing Diagnostic Performance

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Webber, Richard L.	Senior Dental Surgeon	CI	NIDR
	Nagel, Roger	Senior Staff Fellow	CI	NIDR
COPI:	Wiebe, John D.	Sr. Asst. Dental Surgeon	IR	NIDR
COOP:	Reese, Joyce A.	Health Scientist Adm.	RMP	NIDR
	Duckworth, John			NIDR
	Wagner, Robert		BRH	
	Fewell, Thomas		BRH	

COOPERATING UNITS (if any)
Clinical Dental Services Section, NIDR; NIDR Extramural Restorative Materials Programs; Division of Electronic Products; Bureau of Radiological Health.

LAB/BRANCH
Clinical Investigations Branch

SECTION
Diagnostic Methodology Section

INSTITUTE AND LOCATION
NIDR, NIH, Bethesda, Maryland

TOTAL MANYEARS: 1.49	PROFESSIONAL: .77	OTHER: .72

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Factors influencing the clinical performance of dental x-ray systems including a portable dental fluoroscope are being evaluated in vitro using computer simulations and quantitative measurements derived from radiographic phantoms.

Measures of clinical utility based on an endodontic task using dentulous skulls are being determined for various film/screen combinations in order to access the minimum information capacity required. It is anticipated that these findings will aid in establishing the minimum signal-to-noise ratio and resolution characteristics necessary for endodontic radiography.

Other studies based on spectral analyses of ^{125}I and ^{153}Gd using a non-linear systems model indicate significant technical limitations which minimize the practicality of such isotopes as x-ray sources in routine dental fluoroscopic applications.

Factors influencing geometric registration of successive radiographic projections are also being explored in an effort to establish a practical means for quantitatively tracing degenerative changes in tissues of dental interest over time.

Project Description

I. Objectives

The purpose of this project is to integrate theoretical and practical information currently available into prototype dental radiographic systems which can be optimized for specific diagnostic tasks. Emphasis is directed toward improving efficiency by reducing dose and turn-around time for applications requiring less than maximum information capacity. Another objective is the ability to reproducably align the radiographic source and detector relative to the tissues of interest to facilitate longitudinal analysis of destructive lesions.

II. Methods

Promising x-ray sources and image-detector configurations are being developed and evaluated in collaboration with other Government agencies and private interests. The idea is to more rationally relate system elements to the diagnostic task to be accomplished.

Previous efforts included consideration of sealed sources produced from ^{125}I to provide x rays from inside the mouth as originally described by Henrikson. A series of in vitro experiments with sources of this type were undertaken with model systems to determine the effect of source size, intensity, and projection geometry on images of clinical interest. Spectral characteristics were analyzed by computer simulation to determine impact on signal-to-noise ratio and exposure modulation.

Another investigation involves analysis of various film/screen combinations which have been sensimetrically classified in terms of number of noise-equivalent quanta required to produce an image of comparable quality. These detectors are being used to record in vitro images from dental phantoms undergoing endodontic instrumentation in an attempt to determine the minimum radiographic information capacity required to reliably perform such procedures in a routine fashion.

Various experts including technically knowledgeable medical physicists, radio-nuclide engineers, and transducer technologists have been consulted as a prelude to specific efforts directed toward optimization of a practical fluoroscopic system.

Efforts toward geometric optimization have evolved from discussions on the application of feed-back control theory to optimization of exposure geometry which were elicited at a workshop held May 16, 1978 at the University of Connecticut. Efforts are currently underway to explore the technical feasibility of feed-back and/or multiple projection systems via a suitable request for an extramural research contract or interagency agreement.

III. Major Findings

Extensive measurements and computations using ^{125}I as a sealed point-source of x radiation for fluoroscopic purposes have shown that the finite half-life (56 days) coupled with the fall-off in output associated with collimations suitable to yield a reasonable effective "focal spot" preclude image-quality sufficient for all but a few clinical applications irrespective of the gain of the detector. This conclusion also follows from computations based on the use of an extended source having output focused by a suitable collimator. Even if activities of 300 millicuries could be routinely produced in a suitable package free from short-lived high-energy contaminants, shielding and handling such an intense source would be problematical.

Computer simulations using a non-linear attenuation model suggest that the spectra associated with ^{125}I and ^{153}Gd while optimal for certain tasks are not generally well suited to fluoroscopic applications even when an allowance is made for optimal spectral sensitivity of the detector. This is particularly true for ^{153}Gd which in addition to energy in the optimal range (24 - 45 keV for caries detection-tasks) also produces a significant high-energy peak near 100 keV. More conventional (broad-spectrum) sources were shown to be less sensitive to the normal range of attenuations encountered in tissues of dental interest.

As an alternative modified rod-anode sources are being considered which are potentially safer and more flexible in this regard. These devices also exibit a potential for miniaturization made possible by high-speed detection technology, which minimizes the power-handling requirement of the source.

Previous work has demonstrated that an estimated reduction in signal-to-noise power ratio of 30% does not significantly influence the detectability of induced incipient enamel lesions, and preliminary findings suggest that the same will hold for other diagnostic tasks of dental interest, such as the determination of the height of interseptal bone relative to the cemento-enamel junction of adjacent teeth. Hence the use of faster detectors permits considerable latitude in automation of beam-angulation via an on-line feed-back control system or through the use of multiple exposures with no increase in dose to the patient relative to the status quo.

IV. Significance

Taken en masse these findings suggest that the ultimate design of a practical dental fluoroscope will probably not include an isotopic source. A suitable miniaturized rod-anode type intra-oral x-ray tube provides a promising alternative which is currently being investigated for this application.

The use of an automated system for reproducing projection geometry from one x-ray examination to the next would permit relatively small changes in tissues to be accurately and conveniently determined. This is particularly important for interceptive treatment of largely irreversible, degenerative processes such as caries and periodontal disease.

V. Proposed Course

Future efforts will continue to emphasize the multidisciplinary approach described above. Collaborative efforts will be coordinated in ways which optimize the resources conveniently available from the many sources and agencies having interests in this area.

Publications:

Lieberman, J. E. and Webber, R. L.: Clinical evaluation of prototype intraoral source x-ray system. Oral Surg., Oral Med., Oral Path. 46: No. 2, 318 - 327, 1978.

Yin, L. I., Trombka, J. I., Seltzer, S. M., Webber, R. L., Rennie, J., and Farr, M. R.: The lixiscope. SPIE Conf. Proc. 143: 106 - 113, 1978.

Webber, R. L., and Nagel, R. N.: Proceedings from Workshop on Feedback Control of Exposure Geometry in Dental Radiography. (In Press)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 DE 00158-05 CI

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)
Cephalometric Description of Growth Processes Through the Use of Symmetric Axis Coding

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Webber, Richard L.	Senior Dental Surgeon	CI	NIDR
	Nagel, Roger	Senior Staff Fellow	CI	NIDR
COPI:	Blum, Harry	Physical Scientist	LSM	DCRT
COOP:	None			
OTHER:	None			

COOPERATING UNITS (if any)
DCRT, NIH

LAB/BRANCH
Clinical Investigations Branch

SECTION
Diagnostic Methodology Section

INSTITUTE AND LOCATION
NIDR, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.75	.70	1.05

CHECK APPROPRIATE BOX(ES)
☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A geometric method for isomorphically transforming the shape of biological structures expressed as two dimensional projections is being applied to tracings of cephalograms to determine constraints underlying growth and development. The rationale underlying this effort is based on the assumption that the relatively small amount of systematic progress in quantitative morphology may be attributed to a lack of coordinate independent bases for mathematical description.

Symmetric-axis geometry provides a promising alternative to conventional cephalometric descriptions which has lead to the discovery of fixed angular relationships in lateral projections of the human mandible. The associated angles appear to be independent of age within and between individuals. Preliminary findings applied to the mandibular shape of other species suggest that homologous angles may be invariant likewise.

PHS-6040
(Rev. 10-76)

Project Description

I. Objectives

Characterization of the shape of biological structures which grow in predictable ways is difficult using conventional techniques since most common mathematical tools are based on Euclidian geometry which does not take into account developmental redundancy. Attempts at systematic description are often complicated by coordinate-dependent transformations which yield different results depending on the arbitrary selection of fiducial references. A coordinate-independent transformation originally described by Blum permits the shape of closed forms which grow incrementally in time to be efficiently described by means of a central, as opposed to peripheral pattern called the symmetric-axis. The result is a new geometry which capitalizes on the redundancy of linerar growth processes and therefore provides an efficient way to describe shapes which are of interest in studies of biological growth and development. The purpose of this investigation is to continue to explore the utility of this new geometry in the description of biological shape, and to use it as a tool to study factors constraining growth and development in controlled biological experiments.

II. Methods

Any closed continuous plane curve can be expressed as an isomorphic transform which relates all points to a unique central description called the symmetric-axis. This axis has two components;

> The locus of center points of maximally inscribed discs, called the axis points, and the ordered collection of disc radii, called the radius function.

Algorithms have been implemented which permit a digital computer to transform a given two-dimensional figure into its unique symmetric axis representation and conversely reconstruct it from its symmetric axis description in a reversible fashion. Additional algorithms have been constructed to analyze the symmetric axis components and produce a set of shape descriptors. These descriptors provide a rich environment based on the symmetric axis which permits quantitative analysis of the biological shape under study. By comparing these shape descriptors obtained from two dimensional projections of biological structures recorded at various stages of development in man and homologous species it is possible to identify and analyze processes underlying biological growth and development.

III. Major Findings

An investigation of a variety of lateral cephalometric projections of human mandibles including normals abstracted from the Broadbent-Bolton series from age 1 through adult, a longitudinal series obtained from two different patients afflicted with severe untreated mandibulofacial dysostosis (Treature-Collins syndrome), and an isolated example of a Polynesian anomaly known as a "rocker-jaw", has conclusively demonstrated the presence of an invariant angle between branches of the symmetric-axis determined by the junction of three axis-segments near the mandibular foramen. This finding has now been independently confirmed by another investigator on different data using a different symmetric-axis algorithm thus providing a reasonably secure basis for generalization.

Preliminary findings based on a cross-sectional analysis of lateral projections of rat mandibles suggest that a similar invariance may also occur in this animal although the sizes of the invariant angles are likely to be different from those seen in humans. Other preliminary data derived from a study of homologous radius functions associated with symmetric-axes near the angle of the mandible (gonial region) suggest that the curves determined by the upper and lower boundaries of the mandible in this region are relatively parallel.

New methods for analyzing shape from symmetric-axis descriptors have been devised which permit meaningful subdivision of shape into component elements which can be studied individually. As described last year these include methods for studying differential growth-rate within a composite structure. Other promising schemes involve cannonical description of deformations caused by encroachment or flexure, wherein area and periphery of the projection of interest are preserved.

IV. Significance

The discovery of a precisely defined morphological invariant property which follows directly from symmetric-axis description of homologous projections of the human mandible provides a solid justification for future application of this new analytical tool. The fact that this invariant pattern was discovered on the first application of the method to developmental data which can be meaningfully expressed as a two-dimensional projection suggests that other invariant properties are likely to emerge from comparable analysis of other biological structures characterized by similar growth dynamics. Certainly the fact that a relatively invariant angle exists in the jaw implies that certain aspects of mandibular growth and development can be reliably predicted. This necessarily reduces the number of possible alternatives that an Orthodontist must consider when taking into account the role of mandibular growth in his treatment plan.

V. Proposed course

Efforts will be extended to determine factors underlying the observed angular invariance with emphasis on experiments designed to discover conditions for which it does not hold. Other biological structures will be studied to determine the extent to which it may apply to other areas and/or structures. To this end a more efficient algorithm developed by Bookstein at The Center for Human Growth and Development, The University of Michigan will be implemented on a mini-computer to facilitate application of this methodology in our laboratory.

Publications:

Blum, H. and Nagel, R. N.: A Symmetric Axis Basis for Object Recognition and Description. *Pattern Recognition* (1979)

Webber, R. L., and Blum, H.: Angular Invariants in Developing Human Mandibles. *Science* (In Press)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 DE 00211-03 CI

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Enhancement of Diagnostic Images

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Webber, Richard L.	Senior Dental Surgeon	CI	NIDR
	Nagel, Roger	Senior Staff Fellow	CI	NIDR
OTHERS:	Dwyer, Andrew J.	Staff Radiologist	DRD	CC
	Cramer, Harry J., Jr.	Staff Radiologist	DRD	CC

COOPERATING UNITS (if any)

Diagnostic Radiology Department, Clinical Center

LAB/BRANCH
Clinical Investigations Branch

SECTION
Diagnostic Methodology Section

INSTITUTE AND LOCATION
NIDR, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 1.31	PROFESSIONAL: .60	OTHER: .71

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project is an extension of previous work involving the creation, development, and testing of image processing techniques designed to improve diagnostic performance. Recent emphasis has centered on position-invariant filtering schemes to eliminate known sources of noise in CAT scans. Other work involves testing of the computer implemented transformation which creates an isometric three-dimensional "psuedo-solid" from any two-dimensional array of grey levels. Comparisons of processed and controlled images by unbiased observers suggest that diagnostic performance is limited by sample restrictions imposed by a severe angular dependence on the pseudo-solid algorithm when using shadows as cues to depth. Modifications of this and the other promising enhancement schemes reported on last year are being explored to further define factors limiting diagnostic performance.

This project will continue to forgo investigation of empirical enhancement schemes per se in favor of approaches which are based on relatively specific theoretical concepts.

Project Description

1. Objectives

This project is concerned with further study of factors underlying diagnostic performance obtainable from images with emphasis on the effect of mode of display on performance by human interpreters. Previous work has shown that image processing can improve diagnostic performance obtainable from dental radiographs for specific tasks not limited by the information capacity of the system. This work also demonstrated that monocular cues to depth can be meaningfully used to augment perceptibility of complex images of diagnostic interest.

Current objectives are based on logical extensions of these findings with emphasis on performance testing of promising enhancement schemes.

II. Methods

The enhancement schemes described last year are being refined and made available for computer implementaton in ways which facilitate formal evaluation of their impact on diagnostic performance by human observers. This involves developing methods for automating image transformation and display as well as establishing research paradigms which facilitate unbiased acquisition and statistical analysis of performance data obtainable from human observers.

Soft ware has been written which permits multiple displays to be viewed and manipulated simultaneously on a single video monitor. Other programs permit well-defined "lesions" to be induced analytically via computer simulation so that performance can be based on explicit measures of diagnostic utility.

One such program permits an actual dental radiograph to be used to create a clinically meaningful attenuation model of the teeth and related soft tissues. The output of this program is a grey-level look-up table which permits a radiograph taken under one set of conditions (source spectrum, filtration, detector sensitivity, detector response characteristic) to be mapped into the simulated result of exposing the same tissues under another set of user-selected conditions with the same exposure geometry. This program thus permits realistic simulation of ideal x-ray systems as well as providing for enhancement of tissues having known x-ray attenuation characteristics.

Statistical parameters used to evaluate diagnostic performance include measures of signal detectability (ROC analysis) and transinformation as well as a tally of diagnostic errors. A communications link has been established which permits CAT scan data to be accessed directly on a mini-computer so that enhancement studies can be performed with no loss of diagnostic information from the source.

III. Major Findings

Extensive analysis of the detectability of induced dental lesions using the pseudo-solid enhancement technique suggest that the selection of proper angle for illumination relative to the simulated contour is critical. Even a relatively small change in angulation can have a tremendous effect on the size, number and configuration of resulting shadows. This poses few conceptual problems if the angle of illumination is manipulated in an on-line fashion. However, the extensive computations involved precludes this for a practical system at the current state of the art. What appears to be needed is a cue to depth which is less sensitive to illumination geometry, so that less searching is required before a meaningful conclusion can be reached.

Theoretical analysis of factors known to influence the amount of information available from CAT scans made on second-generation machines suggests that visual interpretability may be enhanced by a suitable position-invariant spatial-frequency filter coupled with a context-dependent, contrast enhancement technique (histogram equalization). Preliminary observations based on the use of such a scheme are encouraging but require confirmation by statistical analyses of the performance measures cited above.

Application of the simulation program to model x-ray systems having a detector characteristic with a uniform (linear) contrast gain (gamma) characteristic has produced results which suggest that an idealized detector with these characteristics might be useful in dental radiography. As simulated, a lesion characterized by a fixed difference in attenuation would exhibit the same contrast irrespective of the absolute attenuation of the tissues involved. Hence, under these conditions a comparatively small carious lesion would likely be as easy to see in a molar as in an incisor.

IV. Significance

For the most part it is too early to determine the ultimate significance of this project since most of our efforts to date have been preparatory in nature. Only recently have we actually undertaken pilot experiments designed to measure diagnostic performance obtainable from enhanced images. However, the methodology is well established and has yielded promising findings particularly with regard to diagnostic tasks of dental interest. The theoretical rationale underlying this project is based on systematic assessment of image processing within the context of a complete diagnostic system. Hence, the number of pertinent alternatives to be explored are reduced a priori to a manageable number thus increasing the likelihood of meaningful results.

V. Proposed course

Futher activity will emphasize formal evaluation of selected enhancement techniques within the context of specific diagnostic tasks. It will include an effort to develop a generalized diffuse reflectance model to be used in association with the pseudo-solid transformation to facilitate contour perception which is less sensitive to illuminant geometry than the projection of hard shadows. Theoretical analyses of ideal x-ray systems will continue with emphasis on the perceptual impact of ideal detectors.

Publications;

Webber, R. L., and Nagel, R. N.; Image preprocessing as an aid to visual interpretation. IADMFR Proceedings (1978) (In press)

Webber, R. L. et al.: Three-Dimensional Enhancement of Two-Dimensional Images. Journal of Clinical Engineering. (In press)

Webber, R. L.: On Modeling Dental Radiographic Systems. Proceedings of the Bureau of Radiological Health Symposium on Biological Effects and Dosimetry of Ionizing Radiation. (In Press)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 DE 00048-08 CI

PERIOD COVERED

October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Anatomical Studies of the Head

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Bosma, James F.	Chief, Oral Pharyn.	CI	NIDR
COPI:	None			
OTHER:	Murayama, Diane	Illustrator	CI	NIDR

COOPERATING UNITS (if any)

Pierce, Robt. H., M.D., Dept. Radiol., Univ. Ill., Rockford
Mainen, Michael W., M.D., Practice, Long Prairie, Minn.
Munger, Bryce, M.D., Dept. Anat., Penn. State Univ., Hershey
Schon, Miguel, Ph.D., Dept. Anat., Johns Hopkins Univ.
Bartner, Howard, MA, MAPB, NIH

LAB/BRANCH

Clinical Investigations Branch

SECTION

Oral and Pharyngeal Development

INSTITUTE AND LOCATION

NIDR, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This is the eighth year of anatomical studies of the face, pharynx and cranium of the human fetus at term. The Cranium of the Newborn Human: An Atlas of Tomographic and Anatomic Sections under authorship of Robert H. Pierce, Michael W. Mainen and James F. Bosma was published by the U.S. Government Printing Office in 1977. It has been distributed to prospective reviewing journals and is on sale by the Superintendent of Documents, U.S. Government Printing Office.

A general anatomy book, The Head of the Human Infant, is in preparation of text matching the completed illustrations. Portions of the book are now in critical review.

A collaborative contract project with Dr. Bryce Munger for a study of development of oral mucosal sensory receptors in the rhesus is completed with a comprehensive report, now available through the National Technical Information Service.

PHS-6040
(Rev. 10-76)

Project Description

I. Objectives

To provide a base of descriptive anatomy of the human infant and child for studies of normal development and for definition of malformations.

II. Methods

Routines of dissection and illustration which are standard in the field of anatomy, supplemented by specialized radiography.

III. Major Findings

Publication of book: The Cranium of the Newborn Human: An Atlas of Tomographic and Anatomic Sections. This includes matching tomograms and anatomical sections at 27 levels in the coronal plane, 17 levels in the transverse plane and 25 levels in the sagittal plane.

Preparation of book: The Head of the Newborn Infant. This general descriptive anatomy is in the process of expansion of its original manuscript, with additional illustrations, to total of approximately 240, and corresponding enlargement of text. Its illustrations are essentially completed. Its critique is simultaneously in progress.

IV. Significance

The mouth, nose, pharynx and larynx of the infant differ from these areas in mature man. These differences are in gross form and proportion as well as in detailed anatomy. The descriptions afforded by this project are basic to definition and understanding of postnatal development. Such information is requisite for description of abnormalities of development, including those originating in the peripheral structures and those which evolve secondarily in these structures as effects of central neurological disease.

Such information is also pertinent to description of a variety of diseases and disorders which are acquired in infancy. As example, the evaluation of masses, inflammatory or neoplastic, must be in pertinence to the normal anatomy of the adjacent area in the infant. The normative radiographs of The Cranium of the Newborn Human have already proven to be strategic for this purpose.

V. Proposed Course

Publication Prospectus of a book, The Head of the Human Infant, has been submitted to a publisher, Springer-Verlag.

Publications:

Pierce, R.H., Mainen, M.W. and Bosma, J.B.
The Cranium of the Newborn Infant: An Atlas of Tomography and Anatomical Sections. U. S. Government Printing Office, 1978

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 DE 0012406 CI

PERIOD COVERED
October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Oral Reflexes of the Infant

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Weiffenbach, James M.	Research Psychologist	CI	NIDR
COPI:	None			
OTHER:	Cowart, Beverly	Psychologist	CI	NIDR

COOPERATING UNITS (if any)

Department of Pediatrics, National Naval Medical Center, Bethesda, MD

LAB/BRANCH
Clinical Investigations Branch

SECTION
Oral and Pharyngeal Development

INSTITUTE AND LOCATION
NIDR, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: .60	PROFESSIONAL: .30	OTHER: .30

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☒ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The discrete elicited lateral tongue movement of the human newborn first described under this project continues to be used in psychophysical studies of oral sensation. The transverse tongue reflex described previously can be elicited by separate 5 microliter drops of fluid. The reflex adapts to repeated fluid elicitations. In earlier studies, the response returned when glucose in suprathreshold concentration was added to the stimulus fluid after adaptation to water. This experimental sequence thus indicates taste discrimination. Current studies address the question of sensitivity to the salty taste and its relation to sensitivity to sweet.

Project Description

I. Objectives

This project explores the oral sensory competence of the newborn human infant. A novel reflex technique has been developed as part of this project. It is equally well suited to measuring the local sensitivity of the tongue to touch and to taste stimulation. In the beginning, project investigations were primarily concerned with developmental changes in touch sensitivity. Progressively greater emphasis is now being placed on studies of the sensory mechanisms underlying neonatal taste experience. Since infants are sensitive to taste at birth it is possible, as has been contended on the basis of animal studies, that early taste experience shapes later food preferences and dietary habits.

II. Methods

An easily observed reflex displacement of the tip of the newborn's tongue toward the side of stimulation serves as the basis for assessing the sensitivity of the tongue to both touch and taste. For touch stimulation, the frequence of response occurrence is a function of stimulus intensity. An analogous relation of strength of taste stimulation (concentration of tastant in solution) to frequency of response is obtained providing that responding to the solvent alone has been first reduced by repeated stimulation. Postadaptation increments in responding above that found with continued water stimulation have been demonstrated for both glucose and sodium chloride test solutions.

III. Major Findings

A procedure in which each subject is exposed to both control and test conditions failed to produce the expected results. Response to saline after sucrose adaptation yielded only marginally higher responding than that eliciting by continued sucrose stimulation. Sucrose tests after saline adaptation elicited responses similar to continued saline stimulation. These results appear to reflect interference of the first trial with the second since the first trial data show the expected between subjects effect (sugar after salt > salt after salt and salt after sugar > sugar after sugar). Earlier studies in which subjects received the same sequence of adapting and test fluids on trials one and two failed to show more than minimal interference.

IV. Significance

Studies of neonatal taste in the human, and of the normal development of taste in infancy and childhood will make an important contribution to understanding the development of those destructive food intake patterns that have been implicated in the etiology of caries. Investigations of the impact of "normal" variation in the pattern of early taste experience on later food preferences should lead to the design of effective intervention strategies.

V. Proposed Course

Alternative means of achieving test and control data from the same subjects will be developed and tested with the end of providing a clinically useful individual difference measure of taste sensitivity.

Publications:

Weiffenbach, J.M., Daniel, P.A. and Cowart, B.: The Development of Saltiness Perception. In Fregley, M.J., Bernard, R.A. and Kare, M.R. (Eds.) Biological and Behavioral Aspects of Salt Intake, New York, N.Y., Academic Press, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF
HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DE 00181-04 CI

PERIOD COVERED

October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Postnatal Development of the Rat Skull

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Bosma, James F.	Chief, Oral Pharyn.	CI	NIDR
COPI:	None			
OTHER:	Murayama, Diane	Illustrator	CI	NIDR
	Sapperstein, Selma	Secretary	CI	NIDR
	Koback, Ann	Secretary	CI	NIDR

COOPERATING UNITS (if any) Extramural Programs, National Library of Medicine
Baer, Melvyn J., Dept. Orthod., School Dent., Univ. Mich.
Ackerman, James L., Dept. Orthod., Phila. Child. HOsp. and School of Dent. Med., Univ. Penna.

LAB/BRANCH

Clinical Investigations Branch

SECTION

Oral and Pharyngeal Development

INSTITUTE AND LOCATION

NIDR, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.70	.30	.40

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A PHS Grant Application has been submitted to the National Library of Medicine for subvention of publication of the book, Postnatal Development of the Rat Skull.

In Part 1 of the book, the cranium and mandible of the rat are demonstrated by drawings and photographs at successive postnatal ages. In Part 2, development of the cranium and mandible are demonstrated by alizarin vital staining and photographs of sections. This combination of methods affords comprehensive demonstration of the development of individual bones, of skeletal composites and of the skull as a whole.

Project Description

I. Objectives

Normative demonstration of postnatal development of the individual bones and teeth and of the cranial composite and mandible in the rat.

II. Methods

Of Part 1. Rat skulls of days 1, 15 and 60 were dissected after papainization. The individual bones were then photographed and drawn.

Of Part 2. Rats at 8 postnatal ages were injected alternately with Alizarin Red S or Alizarin Blue BB and sacrificed at intervals after last injection. Defleshed crania and mandibles were mounted in Bioplast and sawn in sections. Patterns of development in separate areas of each bone and tooth and of development of composites of related bones and teeth were worked out by comparison study of specimens stained on various calenders.

III. Major Findings

Normative illustrations are prepared in the cranium and its bones and of the mandible. These constitute the basis of a textual description of the postnatal development of the rat skull on an individual bone and a regional basis.

The patterns of apposition and resorption at the margin and in the interior of the various portions of individual bones and teeth have been defined and further illustrated by schematics. The study materiel of Part 2 as prepared for publication, combines color photographs (in 120 projection slides) with tracings of the corresponding anatomical transsections. Each of these atlas units includes a projection slide, derived illustrations and textual commentary.

This volume also includes a review of vital marking of skeleton by alizarin and other stains which are chemically incorporated into ossifying tissues.

IV. Significance

This is the first comprehensive description of skull development of a laboratory mammal in this extent of age range, in this inclusion of entire skull and all teeth, and in this extent of detail. The descriptions and associated interpretations are significant contributions to information and understanding of normal mammalian development. And they are of normative value in definition of variations of rat skull and tooth development.

V. Proposed Course

A PHS Grant Application, I-R01 LM03464-01, has been submitted to the National Library of Medicine for subvention of publication and sales distribution of this book by the University of Michigan Press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 DE 00182-04 CI

PERIOD COVERED

October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Studies of Sensorimotor Impairments of the Mouth and Pharynx

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Bosma, James F.	Chief, Oral Pharyn	CI	NIDR
COPI:	Weiffenbach, James M.	Research Psychologist	CI	NIDR
	Wolf, Robert O.	Dental Investigator	CI	NIDR
OTHER:	Geoffrey, Virginia	Speech Specialist	CI	NIDR
	Murayama, Diane	Illustrator	CI	NIDR
	Sapperstein, Selma	Secretary	CI	NIDR
	Koback, Ann	Secretary	CI	NIDR

COOPERATING UNITS (if any)

Intramural Programs,
NINCDS and NICMD
Diagn. Radiol. Br., Clinical Center
Dept. of Radiol., Johns Hopkins Medical Center

LAB/BRANCH

Clinical Investigations Branch

SECTION

Oral and Pharyngeal Development

INSTITUTE AND LOCATION

NIDR, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.65	.90	.75

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☒ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Persons who have disorders and/or impairments of feeding, speech, or airway patency are evaluated by standard and cinema photography, and standard and cinema radiography.

Currently, principal attention is directed toward persons who have neurologic impairments of the mouth and/or pharynx. Persons having impairments in this area in association with malformation, cancer or cancer surgery, or inflammatory disease are also under study.

Project Description

I. Objectives

The study of clinical variations which are associated with malformations of inflammatory disease, or of surgical modifications of oral and pharyngeal structure and form, or with peripheral sensory or motor impairments, or with abnormalities of the representations of the oral and pharyngeal performances in the brain.

These studies are designed to define anatomical abnormalities and their physiological correlates. And, for the purpose of devising and applying therapies for disabilities of feeding and of speech and other respiratory functions.

II. Methods

Standard procedures of cinephotography and cineradiography, as well as acoustical and other physiological recordings are employed. The regional performances of feeding, speech and other respiratory actions are described in infants, children and adults. Selected patients having sensory disorders of the oral and pharyngeal regions are also evaluated by sensory testing. Patients who have surgical excision for cancer are studied as examples of the modification of sensory input by ablation.

III. Major Findings

The disorders of feeding and of upper respiratory performances from impairment of peripheral motor performance can be meaningfully described by currently available clinical methods. It is possible to predict certain disabilities of progressive motor disorders. In some circumstances, the impairments are accessible to prevention or to specific therapy.

IV. Significance

Adaptations of selected diagnostic methods, including sensory evaluation, have enhanced the definition of sensorimotor disorders of the mouth, pharynx and larynx. This has contributed to the recognition of related elements of separate disorders, to anticipation of some impairments in progressive diseases, and to therapy of disorders which are shown to have common pathogenic mechanisms.

V. Proposed Course

Clinical studies of subjects having the above categories of disorders will continue with current methods.

Our radiographic studies of sensorimotor pharyngeal impairments are currently under accumulation into a general volume, Radiography of the Pharynx, under authorship of James F. Bosma and Martin Donner, of the Department of Radiology, the Johns Hopkins Medical Center. Publication will be by Springer-Verlag.

Publications:

Bosma, J.F., Physiology of the Mouth
Chapter in press. Otolaryngology, Edition 2. Eds. Paparella, M.M. and Shumrick, D.A., Philadelphia, Saunders Co.

Bosma, J.F. and Donner, M., Physiology of the Pharynx, ibid.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF** **INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 DE 00212-03 CI

PERIOD COVERED

October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Taste and Its Disorders

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Wolf, Robert O.	Dentist	CI	NIDR
PI:	Weiffenbach, James M.	Psychologist	CI	NIDR
	Baum, Bruce J.	Dentist	LMA	NIA
OTHER:	Cowart, Beverly J.	Psychologist	CI	NIDR
	Ellis, Charles	Student trainee	CI	NIDR
	Brown, Vivian J.	Student trainee	CI	NIDR

COOPERATING UNITS (if any)

Hypertension - Endocrine Branch, NHLBI:
Laboratory of Molecular Agency NIA

LAB/BRANCH
Clinical Investigations Branch

SECTION
Oral and Pharyngeal Development

INSTITUTE AND LOCATION
NIDR, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.08	.95	1.13

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☒ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The selection and refinement of appropriate psychophysical methods for the separate measurement of the various aspects of taste perception is a primary and continuing concern of this project. Normal variation in taste perception with chronological age is being assessed by procedures which quantify the intensity and pleasantness of the subject's taste experience. Parallel studies are concerned with anomalous taste experience as a naturally occurring phenomenon or as a consequence of experimental manipulation of diet. Other studies are directed specifically at taste perception difference between individuals with and without caries.

PHS-6040
(Rev. 10-76)

Project Description

I. Objectives

This project employs selected modern psychophysical methods to define the relation of taste perception to age and to oral or systemic disease. Cases in which disorders of taste perception are the presenting symptom are also studied.

II. Methods

Currently, a "sip and spit" procedure is employed to provide whole mouth stimulation with room temperature tastant fluids. All tasting is done with an immediately prior rinse of double-distilled water. For threshold measurement, the "up-down" procedure for determining stimulus presentation order is combined with the forced-choice procedure for measuring response. Measures of the intensity and pleasantness of supra-threshold stimulation are obtained by magnitude estimation and cross modality matching.

III. Major Finding

The sensitivity of magnitude estimation procedures to "sweet aversion" phenomena previously suggested by pilot studies has been confirmed in systematic studies of normal high school and college students.

A novel modification of standard magnitude estimation procedures was perfected which allows subjects to indicate the intensity or pleasantness of their perceptual experience by generating lengths rather than assigning numbers. Children as young as 4.5 years of age, who do not relate numbers to stimulus parameters in an orderly fashion, can be tested with this procedure. Calibration studies with adults indicate that the modified procedure provides more reliable data than that produced when the same individuals respond with numbers. Use of this procedure virtually eliminates "zero" responses that plague the analysis of magnitude estimation data generated in the more traditional numerical version of the task.

Preliminary results suggest that clinical reports of metallic taste may bear a strong relation to the generation of elevated (>100 millivolt) potentials between fillings of dissimilar metal.

IV. Significance

The human taste system that is the focus of this program is a major oral sensory system. Through its role in the control of ingestion, taste affects both systemic nutrition and oral exposure to cariogenic substances. The taste system is liable to dysfunctions which are both debilitating and poorly understood. Variations in taste with systemic disease suggest that they may serve as diagnostic indicators.

V. Proposed Course

Age related changes in the relation of suprathreshold stimulus concentration to the intensity and pleasantness of taste experience are being studied cross-sectionally. Kindergarten age children have already been tested. Additional groups of elementary and high school age subjects and two adult samples will be tested. In a separate, but fully compatible effort, we will collaborate with NIA investigators at the Gerontology Research Center in Baltimore on longitudinal studies of taste and aging.

Studies of the relation of taste to caries status will continue, as will other studies evaluating the reliability and efficiency of clinical assessment procedures. A greater number of patients with taste abnormalities will be accepted for evaluation.

Part B The results of the three compared pancreatic function tests will dictate the continuance of this amylase study.

Part C Studies of the mechanisms of fluoride-induced salivation are planned. Pertinent biological mechanisms to evaluate xerostomia treatment are under consideration.

Publications:

Jam, I., Shoham, M., Wolf, R.O. and Mishkin, S.: Elevated serum amylase activity in the absence of clinical pancreatic or salivary gland disease. Amer. J.Gastroenterol. 70:480-488, 1978.

Henkin, R.I., Lippoldt, R.E., Bilstad, J., Lum, C.K.L., Wolf, R.O. and Edelhock, H.: Fractionation of human parotid saliva. J. Biol. Chem. (in press)

Moutsopolus, H.M., Klippel, J.H., Parlidis, N, Wolf, R.O., Sweet, J.B., Chu, F.C., Sternberg, A.D., Tarpley, T.M.: Correlative histologic and serologic findings of sicca syndrome in patients with systemic lupus and erythematosis. Arthritis Rheum. (in Press)

Wolf, R.O. and Taussig, L.M.: Letter to the editor: Serum Salivary Amylase in Cystic Fibrosis. Pediat. Res. 13: 1076, 1979

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 DE 00217-02-CI

PERIOD COVERED

October 1, 1978 to September 30, 1979

TITLE OF PROJECT (80 characters or less)

Salivary Systems

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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Clinical Investigations Branch

SECTION

Oral and Pharyngeal Development

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☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

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SUMMARY OF WORK (200 words or less - underline keywords)

This project is concerned with mechanisms of production and control of extrinsic (i.e. saliva) and intrinsic (e.g. serum salivary isoamylase) salivary gland products.

A. Human (primarily parotid) saliva chemical consitiuents and mechanisms are evaluated as related to health and disease. Parotid salivary flow rate, protein composition and enzymes (particularly lysozyme and amylase) are evaluated in normals and selected disorders such as Sjogren's syndrome and aphthous stomatitis.

B. The intrinsic secretion of salivary isoamylase in serum of cystic fibrosis of the pancreas and Sjogren's syndrome as well as normal monkey serum and monkey gland homogenates are being studied. Diagnositc application and an understanding of the mechanisms of hyperamylasemia are being sought.

C. Fluoride is being studied as a sialogogue for prevention and alleviation of xerostomic symptoms.

Project Description

I. Objectives

Part A This part of the project involves the study of saliva and associated extrinsic glandular function in normal and diseased states. It includes analysis of normal and abnormal salivary constituents and their effect on oral mucosa. Emphasis has been placed on enzymes and proteins. Parotid salivary lysozyme levels are currently being studied in Sjogren's syndrome and longitudinally during the course of apthous stomatitis (AS) to determine the patterns of variation.

Parotid salivary isoamylases are being studied in cooperation with NIA at the Baltimore City Hospital within a stratified aging population in an effort to identify faster migrating isoamylases which are reported to increase during aging. (Chilla, R. et al 1977).

Parotid saliva and serum from patients with parotid gland tumors were analyzed to determine if parotid salivary amylase and serum amylase can predict parotid gland tumors.

Part B Salivary glands also secrete products internally. This process is analogous to, and sometimes referred to as, an endocrine function. This portion of the project involves the study of this function and associated products particularly in the blood (e.g. serum salivary isoamylase) and its use for diagnosis. In cooperation with NIAMDD, studies of the accuracy and usefulness of the serum pancreatic and salivary amylase ratio obtained by polyacylamide gel electrophoresis (PAGE) as compared to a new and a standard pancreatic function test is in progress.

Part C The mechanisms of stimulation and control of both intrinsic and extrinsic salivary gland secretions are the concern of this part of the project. Subjective clinical evidence indicates that a fluoride (1.23% NaF) mouth rinse can alleviate xerostomic symptoms accompanying head and neck radiation by acting as a unique saliva stimulus. The current study attempts to demonstrate, by the use of a standard fluoride mouth rinse, decreased xerostomic symptoms (i.e., subjective response and flow rate) in patients (head and neck radiation, Sjogren's syndrome, drug therapy) expected to have "dry mouth."

II. Methods

Part A Five consecutive two minute parotid saliva samples are being collected at least weekly from AS patients through at least one complete cycle of ulcers and no ulcers. Lysozyme is assessed by radial diffusion in agar gel containing micrococcus lutens substrate.

Parotid saliva from Sjogren's syndrome patients is assessed for lysozyme levels in comparison with a control group.

Parotid saliva samples from patients with unilateral parotid tumors were quantitatively and qualitatively assessed for amylase and isoamylases respectively. Parotid saliva from the non-tumor side was the within subject control.

Part B Serum and urine pancreatic and salivary amylases were measured by separating the isoenzymes by PAGE and then determining the enzyme activity in serial slices of the gel. Various clinical populations are being assessed.

Part C Administration of a course of fluoride mouth rinse or placebo was assessed as a sialogogue in patients with Sjogren's syndrome, Hodgkins disease treated by radiation, and other therapeutic head and neck irradiation.

III. Major Findings

Part A A statistically significant lower lysozyme level was reported for AS patients during the ulcerative stage of their disease as compared with their non-ulcerative stage.

Elevated parotid salivary lysozyme was found in 17 patients with primary Sjogren's syndrome as distinguished from those with Sjogren's syndrome secondary to hyperlipoproteinemia and with normal control subjects. No difference was found in the total parotid salivary protein among the three groups.

Part B

No statistically significant difference could be found between the total parotid salivary amylase in International Units per liter nor change in isoamylase pattern in the 22 cases of study. In addition there was no consistent pattern of increased salivary or pancreatic fraction of amylase from these patients.

Part C The fluoride mouth rinse was shown to be a sialogogue. The total salivary flow was significantly increased in the 6 person groups of patients having xerostomia in association with Sjogren's syndrome, Hodgkins radiation and other therapeutic head and neck irradiations.

IV. Significance

Part A The etiology of AS is unknown. The decrease of lysozyme in parotid saliva during ulceration in AS patients is a finding without appropriate interpretation. The source of this change is remote from the ulcer site. Herpes labialis of viral etiology is reported to also have decreased lysozyme during ulcerations. The glandular source of lysozyme is thought to be the ductal cells; however, macrophages and monocytes are also known to constantly produce lysozyme. The cytoplasm of granulocytes also contains lysozyme. The explanation for the decrease of lysozyme with ulceration might well be in the dynamics of these populations of inflammation participating cells and in permeabilities of the gland cell membranes.

In primary Sjogren's syndrome the levels of parotid salivary lysozyme are higher than in normal subjects or in those patients who have Sjogren's syndrome secondary to hyperlipoproteinemia. We postulate that the source of lysozyme is from the parotid inflammatory infiltrate and that the assessment of parotid salivary lysozyme may be a diagnostic adjunct for Sjogren's syndrome.

The oral physiological data base of the extensive longitudinal studies of the NAI normative population at Baltimore is increased by the inclusion of the salivary amylase parameters.

Parotid tumors cannot be differentiated by total amylase values in the serum or parotid saliva, by the isoamylase patterns of the serum, by the serum salivary pancreatic ratio, nor by the parotid salivary isoamylase patterns comparing tumor side with non-tumor side.

Part B The diagnostic value of the serum salivary pancreatic ratio obtained by PAGE in cystic fibrosis of the pancreas is being tested. A blood test of this nature would be clinically useful because of difficulty in obtaining duodenal fluid by intubation for pancreatic enzyme testing is difficult in some infants and small children. A new and second test utilizing a test compound of tyrosine bound to PABA is given orally. Normal pancreatic metabolism splits the PABA tyosine band and PABA is found in the urine. No PABA is found in the urine of patients with inadequate pancreatic function. The PAGE serum salivary pancreatic ratio is the third test is an NIAMD study to determine the appropriateness of the three tests.

Part C Fluoride administered as a mouth rinse increases the flow rate in persons xerostomic from Sjogren's syndrome, therapeutic head and neck irradiation or Hodgkin's disease. The fluoride rinse elicits a transient flow lasting as long as 30 minutes after stimulation. This unique silalogogue will help alleviate the xerostomic symptoms which are a universal complaint among these patients. The resulting increase in salivation should diminish the caries liability associated with radiation therapy as well as by direct action of the fluoride on the teeth.

V. Proposed Course

Part A The temporal dynamics of lysozyme in AS parotid saliva are under study. The dynamics of blood lysozyme will next be studied as will the etiology (gene products or complexes) of the isoenzymes of lysozymes previously reported.

Lysozyme in the parotid saliva of primary Sjogren's syndrome patients will also be studied in relation to blood parameters and possible isoenzymes.

The results of parotid amylase analysis will be continued, if meaningful correlations are found in the NAI normative aging population.

We shall not continue study of saliva from parotid tumors.

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